GENETIC DIVERISTY OF RICKETTSIA PARKERI IN SYMPATRIC TICK POPULATIONS AT A FOCAL SITE IN NORTHERN VIRGINIA: IMPLICATIONS FOR PATHOGEN DISPERSAL AND SPOTTED FEVER GROUP RICKETTSIOSIS

By

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ABSTRACT

Rickettsia parkeri is emerging as a tick-borne pathogen of public health importance, particularly for its previously unrecognized role as a potential pathogen responsible for rapidly increasing cases of spotted fever group rickettsiosis in the United States. The primary vectors of *R. parkeri*, *Amblyomma maculatum* ticks, are undergoing a rapid population expansion from their historic range along the Gulf Coast. In 2010, a highly infected (>40%) population of *A. maculatum* was discovered at a landfill in Fairfax County, Virginia. Infected *D. variabilis* were collected the following year, representing a potential spillover event, and ongoing surveillance confirms sustained circulation in this species. Multilocus sequence typing of *ompA*, *ompB*, *sca4*, *gltA*, and the 17 kDa surface antigen precursor gene was used to assess the genetic variability of *R. parkeri* across tick species over time. Analysis confirmed complete sequence identity between *R. parkeri* from *D. variabilis* and *A. maculatum* ticks at the landfill site, while five single nucleotide changes in four of the five gene targets differentiate *R. parkeri* in Fairfax County, Virginia from all sequences available in GenBank. Phylogenetic analysis suggests that *R. parkeri* infection in *D. variabilis* results from the introduction and dispersal of a single *R. parkeri* variant in the region. This study presents novel insight into the transmission dynamics and genetic diversity of *R. parkeri* along the *A. maculatum* expansion front, highlighting the potential for spillover in regions where multiple tick species exist in sympatry.

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INTRODUCTION

Spotted fever group (SFG) rickettsiae are obligate intracellular bacteria vectored by a variety of arthropods, primarily ticks. They are associated with human disease worldwide (Table 1), with the most well-known and lethal of these being *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF). However, SFG rickettsiae range in pathogenicity, and several are believed to be nonpathogenic or to not cause appreciable disease in humans[1]. Historically, RMSF presented as severe febrile illness with a high case fatality rate (>25%), even following antibiotic treatment[2]. In recent years, there has been a marked increase in the number of RMSF cases reported in the southern and mid-Atlantic regions of the United States. This trend is accompanied by an appreciable decline in case fatality to <1% in 2001 that cannot be fully explained by improvements in preventative measures, antibiotics, or supportive care, given the rapid onset of severe disease[2]. In addition, the overwhelming majority of vector ecology studies and ongoing surveillance for SFG rickettsiae in many states report an absence of *R. rickettsii* in field-collected ticks despite increasing case reports[3]. Such trends raise interesting questions about the ecology of Rocky Mountain spotted fever, and suggest that other SFG rickettsiae deserve increased public health attention as the primary etiologic agents of spotted fever group rickettsiosis in North America.

It is important to note that many SFG rickettsiae previously described as nonpathogenic have later been confirmed to cause human infection. *Rickettsia parkeri*, the focus of this study, was one such species. It was commonly described in historical collections of *Amblyomma maculatum* dating back to the 1930s, but was not confirmed a human pathogen until a patient in southern Virginia presented with mild RMSF-like

disease in 2002[4]. Since then, *R. parkeri* has been recognized as an emerging pathogen of increasing public health concern. Upwards of 37 cases of *R. parkeri* rickettsiosis, also known as Tidewater spotted fever, have been confirmed in the United States[5]. However, that number is likely a substantial underestimate due to underreporting[5]. Although generally considered nonpathogenic, several studies have recently suggested that other common tick endosymbionts *R. amblyommii* and *R. montanensis* (vectored primarily by *A. americanum* and *Dermacentor variabilis* ticks, respectively) are able to cause human infection and mild rash[6,7]. Further, cross-sectional studies indicate that over 20% of the population in endemic regions are seropositive for *R. rickettsii*, which greatly exceeds the proportion of the population with a medical history of RMSF[2,8]. There is considerable serologic cross-reactivity in diagnostic tests used to confirm cases of RMSF, and commercial tests are unable to distinguish between different SFG rickettsiae. It has been proposed that many of the reported cases of RMSF may be rickettsioses caused *R. parkeri* and other closely related SFG rickettsiae[6,9]. The Centers for Disease Control and Prevention (CDC) stopped reporting cases of RMSF in 2011 in recognition of this possibility, instead classifying cases with similar pathology as "spotted fever group rickettsiosis"[2].

SFG Rickettsiae Transmission Cycle

Ticks acquire SFG rickettsiae by feeding on *Rickettsia*-infected hosts or by transovarial transmission of the pathogen to offspring. Because of their ability to transmit rickettsiae vertically and transstadially (from one life stage to the next), ticks are considered the primary reservoir for SFG rickettsiae in nature[1]. However, this is controlled in part by the pathogenicity of the bacteria for the tick and transmission

efficiency from female to offspring, and transovarial transmission remains unconfirmed for some tick-rickettsiae systems[1]. In the absence of 100% transovarial transmission efficiency or if infected ticks experience poor survival or fecundity, SFG rickettsiae require additional maintenance in enzootic cycles between wildlife and their arthropod vectors. This is thought to be exhibited by all pathogenic SFG rickettsiae[10]. Humans are

Table 1.1:

Spotted fever group rickettsiae associated with human disease worldwide

Rickettsia Species	Vector	Geographic Region
R. rickettsii	*D. variabilis, D. andersoni*	North and South America
R. parkeri	*A. maculatum*	North and South America
R. montanensis	*D. variabilis*	North America
*R. rhipicephali**	*D. variabilis*	North America
*R. amblyommii**	*D. variabilis, Amblyomma spp.*	North America
R. aeschlimannii	Ticks	Africa, Mediterranean
R. africae	Ticks	Africa, Caribbean
R. akari	Mites	Worldwide
R. australis	Ticks	Australia, Tasmania
R. conorii	Ticks	Europe, Africa, India
R. felis	Fleas	North and South America, Europe, Asia
R. heilongjiangensis	Ticks	East Asia and Russia, northern China
R. helvetica	Ticks	Europe, Asia
R. honei	Ticks	Australia, Thailand
R. japonica	Ticks	Japan
R. massilae	Ticks	Europe, central Africa, Mali
R. monacensis	Ticks	Europe, North Africa
R. raoultii	Ticks	Europe, Asia
R. sibirica	Ticks	Russia, China, Mongolia
R. sibirica mongolotimonae	Ticks	Parts of Europe, China, Africa

Adapted from Azad and Beatd (1998)[10]. Rickettsiae are listed their geographic location and associated primary vector(s). These are specifically listed for pathogens found in North America, and more generally elsewhere. Several species with unknown pathogenicity are indicated by *; those selected are commonly found in North American ticks.

incidental hosts for ticks and "dead end" hosts for rickettsiae, having no role in the maintenance or subsequent transmission of the pathogen[11].

The principle vectors of RMSF are *Dermacentor variabilis* (American dog tick) in the midwestern and eastern United States and *Dermacentor andersoni* (Rocky Mountain wood tick) in the western United States. Previous studies of *R. rickettsii* vertical transmission in *Dermacentor* ticks noted reduced survival and fecundity of infected ticks, as well as reduced vertical transmission by ticks infected during immature life stages[12]. A similar phenomenon is observed in the transmission cycle of other pathogenic SFG rickettsiae; for example, infection with *Rickettsia conorii*, the etiologic agent of Mediterranean spotted fever, results in poor overwintering success in its vector *Rhipicephalus sanguineus*[13]. As in humans and ticks, *R. rickettsii* is also pathogenic to rodent hosts. For successful maintenance of the RMSF transmission cycle, vertical transmission in tick populations and horizontal transmission in an enzootic cycle must supplement each other[14]. Infected adult ticks transmit the pathogen to vertebrates, which serve to infect overlapping generations of ticks[10]. The high pathogenicity of *R. rickettsii* in both host and vector results in an unstable transmission cycle that is highly susceptible to seasonal fluctuations, evolutionary pressure, and other ecological changes. In part, this could explain the low rate of *R. rickettsii* infection observed in ticks in nature (<1%) in recent years[3].

It is thought that *R. parkeri* and other SFG rickettsiae with lower pathogenicity are maintained primarily by transovarial transmission within populations of their natural vector[15]. However, the literature is contradictory in examinations of tick-*Rickettsia* relationships involving presumed "nonpathogenic" bacteria. Studies with *R. montanensis*

and *R. rhipicephali* have indicated both no biological effect on *D. variabilis* and adverse effects in *D. andersoni* when maintained transovarially[12,16]. The ability of *A. maculatum* to maintain *R. parkeri* transovarially, and the effect of the pathogen on tick fitness, is unknown. Laboratory studies have confirmed vertical transmission in an unnatural vector, *Amblyomma americanum* (lone star tick), and in its South American vector *Amblyomma. triste*, making it highly likely that *A. maculatum* is capable of vertical transmission[15,17]. Further, epidemiological evidence from the southeastern US suggests that *R. parkeri* establishes more readily in Gulf Coast tick populations[18]. The rate of infection in several populations is reported at levels higher than expected if the pathogen was only acquired horizontally, or if it exerted detrimental effects on its vector. For example, rates of infection in Virginia and Florida have been reported upwards of 40% and 25%, respectively[18–20].

Transovarial Exclusion of Secondary Rickettsiae

Several seminal studies in Bitterroot Valley, Montana suggested that ticks infected with one SFG rickettsiae were refractory to transovarial transmission of a second species. More RMSF cases were observed on the western side of the valley despite equal presence of rickettsiae infected ticks; later analysis determined that *R. rickettsii* dominated that side of the valley, while ticks on the east side were highly infected with a second agent. It was determined that growth of this "East side agent" in *D. andersoni* is restricted to the ovaries, with no detection in hemolymph surveys, and that it was both transstadially and transovarially maintained[21]. Burdorfer et al speculated that the "East side agent"—later identified as *R. peacockii*—interfered with transmission cycle and maintenance of *R. rickettsii* in nature.

Transmission studies confirmed that ovarial infection with *R. peacockii*, *R. montanensis*, or *R. rhipicephali* was able to inhibit transovarial transmission of *R. rickettsii* in Dermacentor ticks[14,16]. Transovarial exclusion, or interference, has been further demonstrated using "nonpathogenic" bacteria alone: *R. montanensis* blocks the transovarial transmission of *R. rhipicephali* in *D. variabilis*, and vice versa[16], and exposure to the pathogen is accompanied by tissue-specific gene expression in the vector ovaries[22]. Although much remains to be understood about the biological mechanisms behind transovarial exclusion, this indicates a role for tick-derived molecules in invasion of tick cells and prevention of superinfection in tick ovaries[16].

In combination with the detrimental effects of *R. rickettsii* and other pathogenic SFG rickettsiae in ticks, it has been suggested that transovarial interference provides a mechanism for selective sweeps that result in the establishment and maintenance of more benign rickettsiae within tick populations[10]. Correlative evidence exists in nature, and suggests that tick endosymbionts might restrict pathogens to certain vectors, even when populations of competent vectors are sympatric. For example, high rates *R. amblommii* infection in *A. americanum* could explain why pathogenic strains are infrequently detected in surveillance studies[23]. However, it is worth noting that there is still some contention in the literature about the epidemiological relevance of transovarial transmission in the perpetuation (or lack thereof) of severe rickettsial disease in nature[24]. Not all studies are in agreement, and some suggest that ecological factors, as opposed to transovarial interference, limit the geographic distribution of pathogen rickettsiae.

Co-Feeding as an Mechanism for Transmission

In addition to the two classic methods of pathogen transmission to the vector (vertical transmission and acquisition from infectious hosts), pathogen transmission can also occur between an infected tick and uninfected tick co-feeding on the same host in close proximity. This can occur even in the absence of systemic infection of the host: pathogens are exchanged between infected and uninfected ticks directly through saliva at shared bite sites, or through blood meals acquired from nearby bite sites[1]. This phenomenon is influenced by pheromones released following successful attachment to a host that encourage the aggregation of feeding ticks[1]. Additionally, some evidence suggests that pathogens can be transmitted to uninfected ticks separated temporally from infected ticks, but feeding at the same bite site[1].

Transmission during co-feeding is most clearly demonstrated by the efficient transfer of tick-borne encephalitis virus between *Rhipicephalus* ticks feeding simultaneously on uninfected hosts. In this particular case, a series of experiments indicate that the host immune response is responsible, with virus transferred between bite sites by leukocytes[25]. The mechanism has also been observed for both *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in co-feeding *Ixodes scapularis* ticks[26]. Several studies have described the transmission of SFG rickettsiae in co-feeding ticks, although the efficiency with which pathogens are transmitted seems to be dependent on the species of bacteria[1,25,27]. Transmission of *R. conorii* between co-feeding *Rhipicephalus sanguineus* on naïve dogs was 92-100% successful, and was also observed in *R. comorii*-immune dogs (up to 28.5%)[27]. Although not as well characterized for SFG rickettsiae in North American ticks, co-feeding transmission of *R. rickettsii* has been

observed in *D. andersoni*[28]. Transmission during co-feeding appears to be a relatively common occurrence with tick-borne pathogens, and likely serves an important secondary role for the maintenance of rickettsial transmission cycles where transovarial transmission is not completely efficient. Further, co-feeding on a shared host could be a potential mechanism for the transfer of tick pathogens between competent vector species and introduction of the pathogen to atypical vectors.

Ticks have overlapping Host Preferences

Although host preferences appear play a part in limiting the overlap of feeding vector species, it appears to be restricted to local ecological scales[29]. A strong degree of host specificity is observed among ticks in nature, and historically ticks were generally considered specialized for distinct hosts. However, ticks likely fall somewhere between host specialist and generalist[1]. Tick species appear to preferentially feed upon a few primary hosts, but this is restricted by host availability to local communities and major host switches are observed across geographic ranges[29]. For example, it is well-known that *Rh. sanguineus* exhibits very strong host preferences for canines, but humans, birds, and small mammals have been reported hosts[30]. Arguably closer to the "generalist" side of the spectrum, *A. maculatum* has been reported on over 71 host species, with preferences dictated only by life stage and host size; these ticks are also frequently reported human parasites[31]. Many other ticks reported to bite humans, including *Dermacentor, Haemaphysalis*, *Ixodes,* and other *Amblyomma* species, exhibit strong preferences (such as the preference of *D.variabilis* for canines) or ecologically restrictive behaviors (in the case of nidiculous species like *H. leporispalustris*). Thus, although frequently identified parasitizing a variety of hosts, the hosts most commonly associated with these species are

limited[1,10,29,32]. Overlapping host preferences further complicate our understanding of SFG rickettsiae transmission cycles in nature.

Amblyomma maculatum Population Expansion

The increased incidence of rickettsial infection in the mid-Atlantic coincides with the range expansion of several tick species known to harbor SFG rickettsiae. Perhaps most notable among these is *Amblyomma maculatum*, the recognized vector of *R. parkeri*. Commonly known as the Gulf Coast Tick, the historic range of *A. maculatum* is restricted to within 150 miles of the Gulf Coast and coastal Atlantic south of the Carolinas[31]. Beginning in the 1970s, isolated populations were described further inland across Oklahoma and Kansas, likely from cattle imported from the Gulf Coast[31,33]. Follow-up surveys on inland populations indicate that individual *A. maculatum* populations have expanded to occupy a continuous distribution across parts of the two states[31]. In more recent years, incidental collections have been reported as far north as Maine[31]. Although the original distribution is considered largely unchanged, *A. maculatum* are reported with increasing frequency in collections across the mid-Atlantic, and it is recognized that the geographic range of *A. maculatum* is undergoing a more general expansion inland and northward[31]. Current surveillance identifies focal, patchy populations of *A. maculatum* across the mid-Atlantic, with reports of established permanent populations in southeastern Virginia in 2010[19]. Such studies highlight the potential for introduced *A. maculatum* to undergo population and range expansion, with subsequent public health implications from the introduction of *R. parkeri* or other pathogens into new regions.

Birds are increasingly recognized as a major force driving the range expansion of ectoparasites and dissemination of rickettsial pathogens worldwide[32,34–36]. Immature *A. maculatum* are known to feed on primarily on rodents and birds[5,31]. A survey of conducted in Louisiana during the northward migration revealed that the majority of ticks parasitizing migratory songbirds were exotic in origin, and over 71% of the ticks were infected with SFG Rickettsiae[36]. Similarly, *R. parkeri*-infected *A. maculatum* ticks are reported in migratory bird infestations along flyways in Maryland[37]. Transport of *A. maculatum* by birds to new geographic regions has been reported as far north as Canada[38].

Mounting evidence from serologic assays and field studies also indicate a potential role for birds as reservoirs, although it remains unclear. No laboratory transmission studies have confirmed the ability of ticks to acquire bacterial infection after feeding on birds, although *R. rickettsii* has been recovered from bird blood following artificial infection[39]. Birds are able to sustain levels of rickettsemia that should be sufficient for transmission of bacteria to ticks[39]; however, this may contribute only occasionally to the transmission of bacteria to ticks, and the literature remains divided about the ability of birds to function as competent reservoirs[32,34,35,39]. Much of the evidence for birds as reservoirs comes from correlative observations of infected host-infected tick associations. Regardless, migratory birds unquestionably have the potential to expand tick ranges throughout North America, introducing and disseminating pathogens in the process.

The Founder Effect and Genetic Diversity

Tick range expansion occurs by spread at the edge of source populations

("pushed" expansion) or by establishment and growth of new populations ahead of the current range ("pulled" expansion)[40]. Modeling and field studies indicate that each imparts a signature on the genetic diversity within populations[41]. Colonization is heavily influenced by Allee effects, or low per-capita growth rates caused by reduced reproductive fitness at low population densities, and only a few members of the initial population persist at a "pulled" expansion front. A new population established by a limited number of individuals results in a non-random sample of genetic information from the donor population and an overall observation of low genetic diversity among members of the new population[40]. One would expect tick colonies initiated by bird drop offs to exhibit this phenomenon, commonly known as the "founder effect".

Lyme disease (agent: *B. burgdorferi*) is arguably the most studied vector-borne disease in North America, and the large amount of available data provide insight into the population genetics of pathogens following tick range expansion. Complex population structure in established populations of *Ixodes* ticks is expected to reflect a number of variables, including host abundance, climate, tick phenology, and habitat, although genetic signatures of expansion are common[40,42]. A longitudinal tick surveillance study on a coastal Maine island provided a unique opportunity to observe sequential *Ixodes scapularis* tick colonization and *B. burgdorferi* introduction in real time. Following *B. burgdorferi* detection in the population, analysis indicated that one founder strain dominated over 91% of samples[43]. Subsequent years noted the gradual replacement of the dominant strain with strains common to the mainland, which the authors hypothesize to be the result of additional introductions of infected ticks from the mainland on migratory birds[43]. Further evidence for the founder effect in *B. burgdorferi* within founding *Ixodes*

populations come from recently established populations in southern Canada and the Midwest[40,44].

A. maculatum ticks are generally thought to exhibit low population structure over their range, a result of mobile hosts and high levels of gene flow between tick colonies[42]. Single-strand conformation polymorphism analyses of *A. maculatum* 16S rRNA from multiple sites in Mississippi indicated genetic similarity between northern and southern populations in the state, and no difference in haplotype frequencies compared to tick populations in North Carolina[45]. In the same study, analysis of *ompA* and intergeneic spacer regions indicated no genetic difference between *R. parkeri* from the two states, although the paper alludes to other data indicating high regional variability[45]. Similarly, Gulf Coast ticks from Texas, Oklahoma, and Kansas are monophyletic and share haplotypes between populations[46]. Both studies also note the presence of haplotypes that are unique to specific geographic regions[45,46]. However, both of these studies were conducted well within the currently established Gulf Coast tick distribution, where interpopulation gene flow is most likely. No studies have directly addressed the diversity of *A. maculatum* or *R. parkeri* in satellite populations, and a need exists for more complete population genetic analysis across the entirety of its suspected expansion.

Surveillance in Fairfax County, Virginia

Routine disease surveillance by the Fairfax County Health Department uncovered a population of *Amblyomma maculatum* infected with *Rickettsia parkeri* at a closed landfill in Fairfax County, Virginia in 2010 (Fig. 1.3). The close proximity to residential neighborhoods and recreational greenspace prompted increased surveillance for tick-borne pathogens in the region. Initial surveys indicated an infection rate of 41.4% in the

A. maculatum population[20]. The high infection rate at the landfill is consistent with reports from other satellite populations in southern Virginia (43-55%)[19], although noticeably higher than rates across the remainder of the southern states (range: 1.5 – 33%)[19,47]. The high infection rate and focal nature of infestations across Virginia are suggestive of maintenance via transovarial transmission in a newly established population, a hypothesis reinforced elsewhere, perhaps following isolated bird drop offs of infected ticks[19,20]. Major highways and fencing around the landfill site partially restrict the movement of large mammals, such deer and cattle, which have been the postulated source of *A. maculatum* introductions elsewhere[33].

At the time of discovery, *R. parkeri* infection appeared to be restricted to *A. maculatum* collected from the site. The ranges of several tick species, including *D. variabilis, Amblyomma americanum*, and *I. scapularis* overlap in the county, and *Rhipicephalus sanguineus* and *Haemaphysalis leporispalustris* are also reported in surveillance collections[48]. As previously suggested, sympatric tick populations provide opportunities for pathogen transfer between tick species. Infected *D. variabilis* have been commonly collected in subsequent years of follow-up at the landfill, beginning in the spring of 2011[20,48]. *R. parkeri* infection in other unusual (*Rh. sanguineus*) and novel (*H. leporispalustris*) ticks has been reported infrequently in the county[20,48]. Geographic and temporal concordance of the infected samples suggest that the presence of *R. parkeri* in *D. variabilis* might be the result of spillover during co-feeding with infected *A. maculatum*[48]. Infection of *D. variabilis* is notable: it has been reported only one other time, and little is known about the ecology, maintenance, or vector competence of the pathogen in this system[49]. If these speculations are correct, the founder's effect

postulates that the *R. parkeri* population at the landfill should exhibit low genetic diversity following invasion of a new geographic area and colonization of resident tick species.

An increase in reported incidence of spotted fever group rickettsiosis and confirmed *R. parkeri* infections in Virginia in recent years underscore the need for further investigation of *R. parkeri* dynamics within tick populations, particularly in light of *A. maculatum* population expansion. Targeted surveillance at the landfill has been ongoing since 2010, and the availability of samples over a period of several years provide a temporally unique dataset for assessing the variability of *Rickettsia parkeri* in a newly established population of *A. maculatum* ticks. Multilocus sequence typing (MLST) was used to investigate the genetic diversity of *R. parkeri* across tick species over time, and phylogenetic analysis was used to infer the relationship between samples. Longitudinal studies of disease ecology provide key information about pathogen persistence and invasion in novel environments. To our knowledge, this represents the first characterization of *R. parkeri* genetic diversity at local scales across a sympatric population of ticks.

CHAPTER I

Genetic characterization of *R. parkeri* in a sympatric population of ticks in Fairfax County, Virginia by multilocus sequence typing

A BRIEF NOTE ABOUT MULTILOCUS SEQUENCE TYPING

The spotted fever group (SFG) within the genus *Rickettsia* consists of a large number of very closely related species, and the phylogenetic classification of members remains an ongoing area of research[50–54]. This is further complicated by the increasingly frequent discovery of novel *Rickettsia* species. Rickettsiae are strictly intracellular, and traditional methods for describing bacterial species based on phenotypic characteristics are not applicable. Antigenic, morphological, and metaboloic characteristics, as well as official molecular criteria based on DNA-DNA hybridization, G+C content, and 16s rRNA divergence used to classify bacterial isolates, do not readily differentiate between members of the spotted fever group[51]. Individual analysis and comparison of 16s RNA genes easily differentiated members of the SFG from other rickettsiae, but intragroup 16s RNA gene sequences are virtually identical (<2% divergence)[55].

Sequencing and analysis of individual genes has vastly improved SFG taxonomy. More recent phylogenetic analyses are dominated by use of the Rickettsia-specific *ompA*[54,56], *ompB*[54,57], and *sca*[50] family genes, which encode high molecular weight surface proteins, and a gene encoding the 17 kDa protein. The most variable *Rickettsia*-specific gene is *ompA* , and it is generally accepted to be the most robust in differentiating between the many members of the spotted fever group[50]. Analysis of this gene supports clustering of SFG species into distinct groups. However, it is absent from some species, and analysis of other targets is necessary. Phylogeny inferred from sequences of the panbacterial gene *gltA* encoding citrate synthase identifies four clades within the SFG, corresponding to an *R. rickettsii*, *R. massiliae*, *R. helvetica*, and *R. akari* cluster[58] (Fig. 1.1). *Rickettsia parkeri*, the focus of this study, groups consistently with

the *R. rickettsii* cluster. Sequences of *ompB* and *sca* genes support the findings of *ompA* analysis to varying degrees, but phylogeny based on 17 kDa protein gene sequences is not statistically supported[51,52].

Multilocus sequence typing (MLST), or the combined analysis of five or more genes, has been proposed as more robust tool for distinguishing between closely related bacterial species[53,59]. Phylogenetic analysis based on MLST is thought to be an accurate representation of relationships inferred from whole genomes if target sequences are carefully selected[53,60]. Both housekeeping (genes required for basic cell function; i.e., citrate synthase) and variable genes, such as antigens, should be used if the goal is to differentiate between closely related strains[53]. This method has improved the accuracy of phylogenetic placement of species within clusters[51,53]. It has also been successfully applied to analysis of single *Rickettsia* species. The diversity of *ompB* and *sca4* readily differentiated between *R. prowezekii* isolates from Africa and North America[61]. Similarly, SNPs in *gltA* and *sca* family gene sequences differentiate between strains of *R. felis*[62].

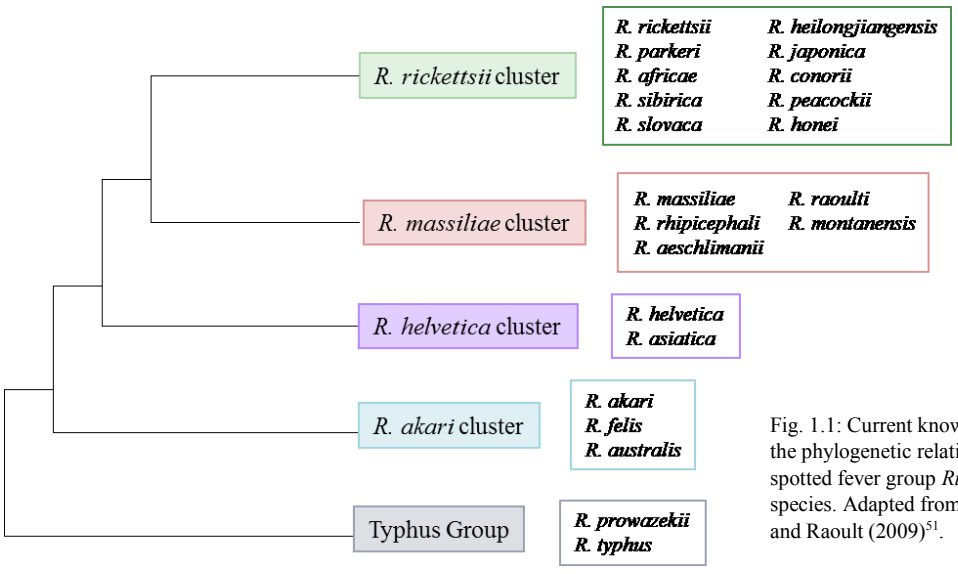


Fig. 1.1: Current knowledge on the phylogenetic relationship of spotted fever group *Rickettsia* species. Adapted from Fournier and Raoult (2009)[51].

In this study, multilocus sequence typing of five targets (*ompA*, *ompB*, *gltA*, *sca4*, and the 17 kDa surface antigen precursor genes) was used to characterize *R. parkeri* genetic diversity at a local scale across a sympatric population of *A. maculatum* and *D. variabilis* ticks. Use of concatenated sequences was analyzed for its capacity to distinguish *R. parkeri* relationships at an intraspecies level at one geographic focus, and for its ability to distinguish *R. parkeri* in Fairfax County from other locations.

MATERIALS AND METHODS

Field Collections

Ticks were collected weekly in 2014 using flagging or drag cloth (March – November) and traps (December – January) by the Fairfax County Health Department at locations in Fairfax County (Fig. 1.2). The vast majority of samples were collected from a site southwest of the I-95 landfill complex. Nine sets of two parallel transects (30 m) were established below a power line closely flanked by wooded areas. During the winter, collections were performed with four CO_2 collection traps randomly placed along the transect area. Limited numbers of additional specimens were collected from Lorton, several miles southeast of the landfill. Ticks were morphologically identified to species, sex, and life stage by the field teams and frozen at -20˚C until DNA extraction. Depending on quantify collected, ticks were maintained individually or pooled in groups of five. Samples from the years 2010 – 2013 were collected using the same methods; DNA was previously extracted and readily available for analysis and pathogen screening. Some of these specimens were obtained from local veterinary clinics and deer hunts in Fairfax County, although this method of sampling was discontinued in 2014.

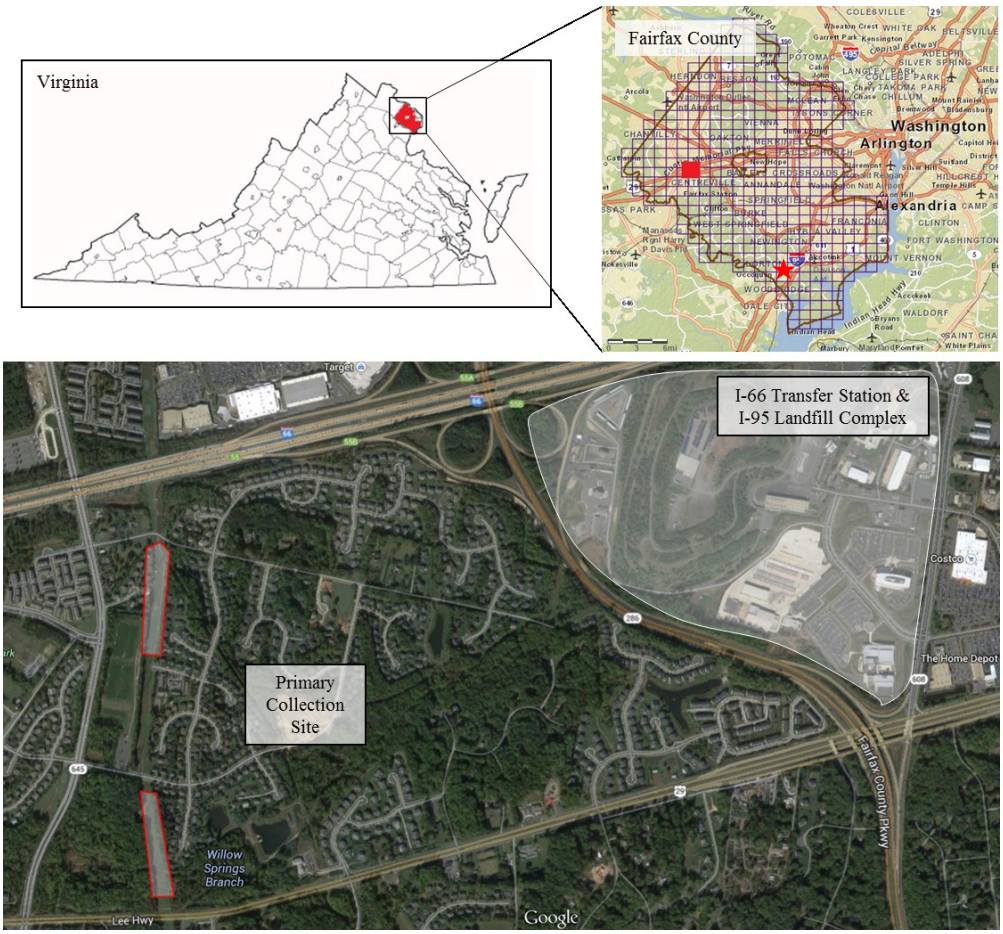


Fig. 1.2: Satellite image of transects at collection site in relation to landfill complex. Red square in Fairfax County insert indicates region in satellite image. The red star indicates Lorton, where additional collections were performed.

Sample Preparation

Genomic DNA was extracted from ticks collected during 2014 using a modified EPICENTRE MasterPure Complete DNA and RNA Purification Kit (EPICENTRE Biotechnologies, Madison WI; see Appendix for protocols) as previously described[48]. Briefly, 5-mm stainless steel beads and lysis buffer was used to homogenize pooled or individual ticks in a TissueLyserII (Qiagen, Valencia, CA). Incubation with proteinase K and lysis buffer solution was used complete lysis, followed by protein precipitation and DNA isolation. PCR was used to confirm extraction quality on a subset of samples using mitochondrial 12S and 16S primers.

Molecular Detection of SFG Rickettsiae

DNA extractions from all *D. variabilis* and *A. maculatum* ticks collected from January – December 2014 were screened for SFG rickettsiae using a direct PCR assay with primers 120-2788 and 120-3599 to amplify an 811-bp fragment of the rickettsial outer membrane protein B gene *(ompB)*[50]. Sequencing of amplicons from *ompB* positive ticks was used to confirm *Rickettsia* species and the presence of *R. parkeri* infection. Samples from 2011 – 2012 had previously been identified as *R. parkeri*- positive using a nested PCR assay amplifying the rickettsial outer membrane protein A gene (*ompA*) followed by PstI endonuclease restriction fragment length polymorphism (RFLP) analysis to confirm rickettsial species[48]. Divergence from previously established protocols was initiated following several ambiguous *ompA* PCR results in screened samples from 2014, but use of *ompB* was confirmed as an equally sensitive and acceptable surveillance tool for SFG rickettsiae.

Multilocus Sequence Typing

Additional genetic characterization was performed on all samples testing positive for *R. parkeri* by using PCR assays targeting four additional genes corresponding to three surface proteins and citrate synthase (Table 1.2). A modified nested PCR assay was used to amplify a 600-bp fragment of the *ompA* gene[57]. The 50 µl inner and outer reactions were improved with increased template DNA (2.0 µl) and increased *Taq* DNA polymerase (2.5 U). The thermocycler program for the inner nested reaction of the *ompA* assay was also modified to include 45 denaturation cycles. A 623-bp fragment of the intracytopasmic protein Gene D was amplified directly using primers D767f and D1390r [50]. For the citrate synthase gene (*gltA*), primers RpCS.877p and RpCS.1258n were used

Table 1.2: PCR assays used in genetic characterization

Assay	Primers	Sequence	Target
rOmp A[57]	190-70 190-701 190-FN 190-RN	5' – ATGGCGAATATTTCTCCAAAA – 3' 5'- GTTCCGTTAATGGCAGCATCT – 3' 5' – AAG CAA TAC AAC AAG GTC – 3' 5' – TGA CAG TTA TTA TAC CTC – 3'	~600 bp fragment of the *ompA* outer membrane protein A gene
rOmp B[50]	120-2788 120-3599	5'- AAA CAA TAA TCA AGG TAC TGT - 3' 5' – TAC TTC CGG TTA CAG CAA AGT – 3'	~800 bp fragment of the *ompB* outer membrane protein B gene
Citrate Synthase[63]	Rp877p Rp1258p	5' – GGG GGC CTG CTC ACG GCG G – 3' 5' – ATT GCA AAA AGT ACA GTG AAC A – 3'	~ 381 bp fragment of the *gltA* citrate synthase gene
17 kDa Surface Antigen[64]	17KD5' 17KD3' 17KD3'nest	5' - GCT TTA CAA AAT TCT AAA AAC CAT ATA-3' 5'-CTT GCC ATT GTC CRT CAG GTT G-3' 5'-TCA CGG CAA TAT TGA CC-3'	~435 bp product of 17kDa cell surface antigen precursor gene
Gene D[50]	D767f D1390r	5'-CGA TGG TAG CAT TAA AAG CT-3' 5'-CTT GCT TTT CAG CAA TAT CAC-3'	~600 bp product of *sca4* ('gene D') antigenic 120kDa surface protein PS120

to amplify a 381-bp segment at the 3' end of *gltA*[58]. A semi-nested PCR was used to amplify a 435-bp segment of the 17 kDa cell surface antigen gene. For each of these assays, RNase-free water was used as a negative control, and DNA extracts of *R. parkeri*, *R. conorii*, or *R. amblyommii* were used as positive control samples.

All amplicons were compared to sequences in GenBank using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information). Only samples with complete sequence information for all five gene targets were included in the phylogenetic analysis. Some sequence information was available from *H. leporispalustris* and *R. sanguineus* that tested positive for *R. parkeri*. Given that these are unusual vector species for *R. parkeri*[48], sequence data was included from these samples when available, although all five gene targets did not successfully amplify.

All publically available *R. parkeri* sequences were included in the data set for comparison. Other *Rickettsia* species from the spotted fever group and typhus group were included when appropriate. Information on the samples and additional sequences are provided in Table S.1 and S.2.

Characterization of Genetic Information

Multiple alignments of individual gene fragments and concatenated sequences of all gene fragments were built using BioEdit[65] and MUSCLE[66]. In the event of nucleotide divergence, translation of sequences into amino acid sequences was performed in BioEdit. Phylogenetic analyses were performed in MEGA6[67] using the maximum parsimony and neighbor joining methods, with statistical support deriving from 800 - 1000 bootstrap replicates. Sequences of other rickettsial species were included for comparison (listed in Table 1). The sequences of typhus group rickettsiae (*R. prowazekii* and *R. typhi*) were selected as outgroups in the phylogenetic analysis, with the exception of phylogenetic analysis based on partial sequences of the *ompA* gene alone, which is absent in these species. In this case, *ompA* sequence from *R. australis* was used as an outgroup.

RESULTS

Frequency of *R. parkeri* Infection in Ticks

A total of 1389 D. *variabilis* were collected in 2014. The prevalence of *R. parkeri* infection in ticks collected from Fairfax County, Virginia was determined by screening samples with *ompA* or *ompB* gene-specific PCR assays. Results from years prior to 2014 are discussed elsewhere, but are summarized in Fig. S1[20,48]. A total of 441 ticks from 2010 – 2014 tested positive for *R. parkeri* (Table 1.3). However, to eliminate concerns about the quality of older samples, the study was restricted to 69 representative ticks testing positive during 2012-2014. During 2014, molecular evidence and maximum likelihood estimates indicate that 1.8% of *D. variabilis* ticks collected from Fairfax

County, Virginia are infected with *R. parkeri*. No infection was detected in any of the nine *A. maculatum* ticks collected during
2014. All positive samples from 2014 were collected from the landfill site.

Characteristics of *R. parkeri* gene sequences.

A subset of 35 seropositive ticks collected from 2012-2014 at the landfill site were selected for genetic analysis. In total, complete sequence information for all five partial *R. parkeri* gene targets was obtained from 11 *A. maculatum* and 22 *D. variabilis* samples (Table S.1). These represent samples from both the landfill and Lorton sites in Fairfax County. Unfortunately, this study was able replicate PCR positivity in only one of the three *Rh. sanguineus* and one of the two *H. leporispalustris*, and complete sequence information is not available for all targets. Sequences for individual targets and concatenated sequences of all targets were compared across samples from Fairfax County and to sequences available in GenBank. Datasets included 58, 45, 41, 36, and 41 partial gene sequences for *ompA*, *ompB*, *gltA*, *sca4*, and the 17 kDa surface antigen precursor gene, respectively. Differences reflect public availability of *R. parkeri* sequences and

Table 1.3: *R. parkeri* infection of ticks in Fairfax County, Virginia

	2010		2011		2012		2013		2014	
Tick Species	**Total (pos)**	**Rate (%)**	**Total (pos)**	**Rate (%)**	**Total (pos)**	**Rate (%)**	**Total (pos)**	**Rate (%)**	**Total (pos)**	**Rate (%)**
Amblyomma maculatum	493 (202)	40.7	547 (169)	30.9	51 (12)	23.53	9 (1)	11.11	9 (0)	0
Dermacentor variabilis	1132 (0)	0	1523 (2)	0.2	2274 (17)	0.75	1087 (8)	0.74	1389 (28)	1.8
Rhipicephalus sanguineus	0 (0)	-	0 (0)	-	60 (2)	3.33	121 (1)	0.83	0 (0)	-
Haemaphysalis leporispalustris	0 (0)	-	0 (0)	-	5 (1)	20.0	92 (1)	1.08	0 (0)	-

Values reflect yearly collection total (*R. parkeri* positive pooled or individual ticks) and infection prevalence (%) at the landfill site.

supplementation of additional sequences from other samples, when available. Subsequent analysis identified five single nucleotide changes in four of the five gene targets that differentiate all *R. parkeri* in Fairfax County, Virginia from other locations, regardless of collection year or tick species (Table 1.4). The full genome of *R. parkeri* strain Portsmouth (GenBank accession no. CP003341) was used as a reference sequence for nucleotide and amino acid counting purposes. All alignments to this reference genome are provided in Fig. S.2. GenBank accession numbers for all sequences are provided in Table S2.

***ompA*.** Complete *ompA* identity was observed between 25 *R. parkeri* sequences from ticks collected at the landfill site and partial sequences from North and South America (U43802, FJ986616, and EF102238, others; see Table S.2). Ten samples exhibit a possible 540C→A nucleotide substitution in the *ompA* sequence. However, the chromatogram trace shows a double peak at this position, with both cytosine and adenine peaks clearly visible. This is indicative of a DNA mismatch or mixed infection. Closer examination across the dataset indicates that other samples might exhibit this feature, but the called base (C) is congruent with publically available sequences. This feature was equally distributed across collection year and tick species. Adenosine at this position seems to be conserved across the remainder of the SFG; the only exceptions are *R. parkeri* and *R. aeschlimannii,* which exhibit C and G at this postion, respectively.

sca4. The amplicon sequence obtained for *sca4* was altered by one nucleic acid when compared to the two available R. parkeri sequences (GenBank accession nos. AF155059, CP003341). Mutation 1377T→C nucleotide change in the *sca4* sequence 'Gene D' is unique among *R. parkeri* from Fairfax County, and results in a V459A

Table 1.4: Informative *R. parkeri* divergence from published sequences.

Target	Gene	Position	Nucleotide Change	Amino Acid Change	# Sequences
Citrate synthase II	*gltA*	145	C→A	Synonymous	All
Citrate Synthase II	*gltA*	508	T→C	Synonymous	All
Citrate Synthase II	*gltA*	342	C→G	$V \rightarrow L_{323}$	2
PS120	*sca4* ('Gene D')	1377	T→C	$V \rightarrow A_{459}$	All
17 kDa surface antigen	17 kDa precursor gene	387	G→A	Synonymous	All
rOmpB	*ompB*	1295	A→G	$V \rightarrow A_{1226}$	All
rOmpA	*ompA*	540	C→A	Synonymous	10*

substitution in the 120 kDa surface antigen PS120. Interestingly, cytosine at this position is conserved across all spotted fever and typhus group sequences examined, with the exception of the two available *R. parkeri* sequences.

ompB. All *ompB* sequences differed from other *R. parkeri* sources (GenBank accession nos. AF123717, KJ158745, CP003341) at one position. The difference was a nucleotide change 1295A→G corresponding to a V1226A substitution in rOmpB. Other species from the SFG are more variable at this position, although species in the *R. rickettsii* cluster appear to favor A at this position.

17 kDa surface antigen gene. All the samples had one synonymous 387G→A nucleotide change in the 17 kDa surface antigen gene that is not shared by any available *R. parkeri* sources (GenBank accession nos. CP003341, U59732, KM245157, others) or any other spotted fever or typhus group rickettsiae.

gltA. The citrate synthase gene proved to be the most divergent in this study. Two synonymous nucleotide substitutions 508T→C and 145C→A are shared by all samples and differ from the remainder of *R. parkeri* sources (GenBank accession nos. CP003341,

KF783219, others). In both cases, other rickettsiae exhibit either of nucleotides (T/C or C/A) at their respective positions in the sequence. Two samples are unique, sharing an additional nonsynonymous mutation 342C→G in *gltA* corresponding to a V323L substitution in citrate synthase. These samples (FXVA2052 and FXVA2927) correspond to seropositive *D. variabilis* and *A. maculatum*, respectively, and were both collected in 2012. No other rickettsiae share this nucleotide change.

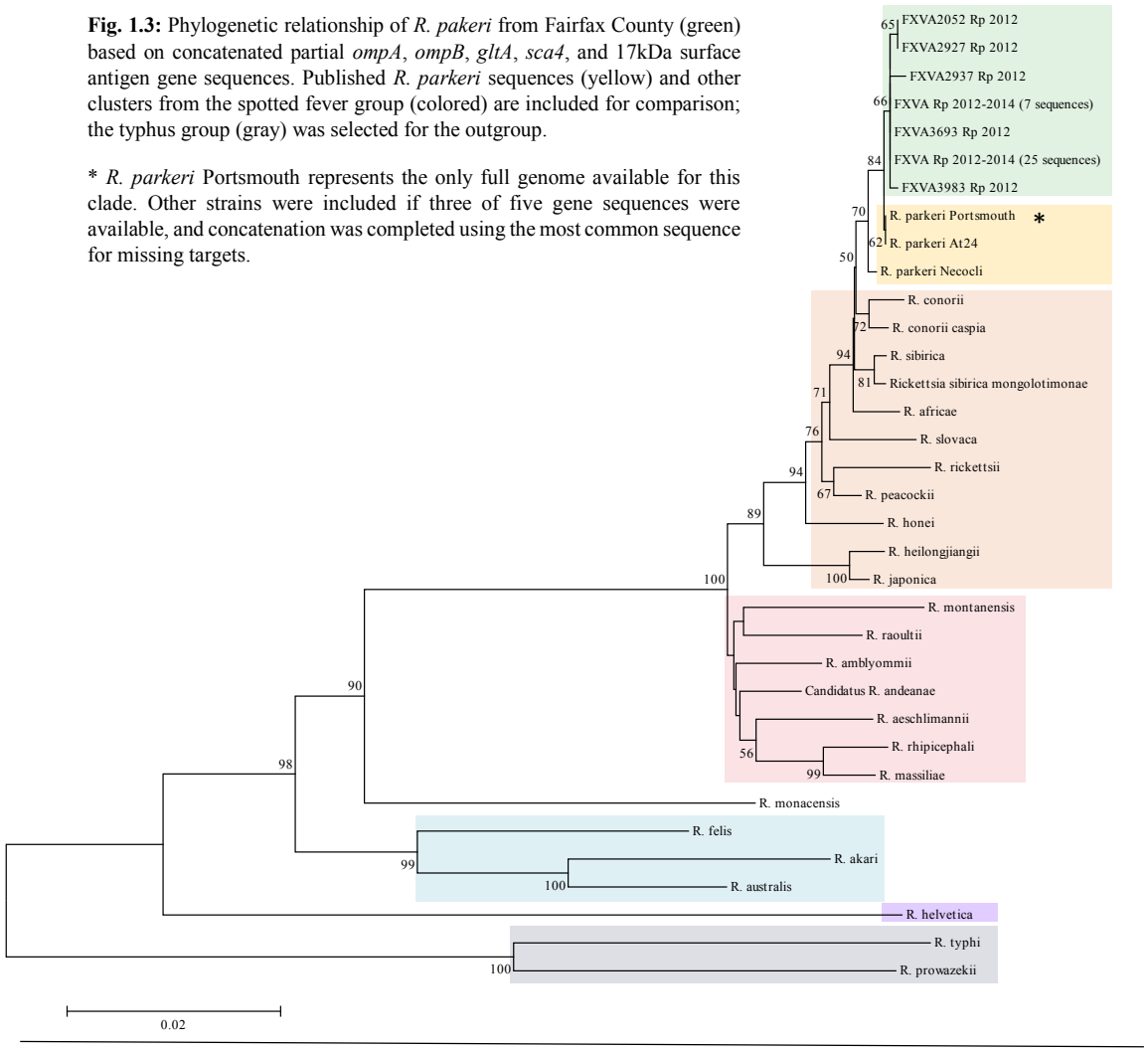
Lastly, several other samples (FXVA4171, FXVA3983, FX2937, FXVA3693) are additionally differentiated by one unique change each in one of the selected genes. All four were collected in 2012. Sample FXVA4171 represents an infected *Rh. sanguineus* tick collected at an animal shelter. One *D. variabilis* (FXVA3983) and two *A. maculatum* comprise the remaining three samples, all of which were collected at the Lorton site.

Phylogenetic Analysis

When the sequences of all five loci were concatenated and compared, two main genotypes were revealed (Fig. 1.3). Twenty-five identical sequences on the all-gene tree correspond to the five shared mutations previously discussed. Seven identical sequences correspond to sequences with a potential *ompA* discrepancy, although this remains ambiguous. To improve the accuracy of phylogenetic relationships, concatenated sequences from other members of the spotted fever group were included. For those species where not all sequences are available from a single genome (i.e., *R. peacockii*), sequences were concatenated from fragments pulled from variety of isolates representative of that species. A neighbor joining approach reveals that all sequences from Fairfax County cluster into a well-supported clade phylogenetically distinct from other *R. parkeri* sequences (bootstrap value of 84%). These groups were also recovered

Fig. 1.3: Phylogenetic relationship of *R. pakeri* from Fairfax County (green) based on concatenated partial *ompA*, *ompB*, *gltA*, *sca4*, and 17kDa surface antigen gene sequences. Published *R. parkeri* sequences (yellow) and other clusters from the spotted fever group (colored) are included for comparison; the typhus group (gray) was selected for the outgroup.

* *R. parkeri* Portsmouth represents the only full genome available for this clade. Other strains were included if three of five gene sequences were available, and concatenation was completed using the most common sequence for missing targets.



in maximum likelihood and maximum parsimony trees (results not shown). Unique *R. parkeri* sequences (FXVA2937, FXVA3693, FXVA4171, FXVA3983, FXVA2927) were all collected at the Lorton site and are distinct from sequences at the landfill. Although the purpose of this study was to characterize the phylogenetic relationship of one species at one geographic focus, it is worth noting that all four clusters within the spotted fever group were well resolved with bootstrap values above 70%. This is consistent with the resolution obtained by others, with the exception of the placement of *R. helvetica*. Previous analyses group *R. monacensis* with *R. helvetica*, and place the pair consistently between the *R. akari* and *R. masilliae* clusters[51].

Single gene phylogenies are broadly compatible with the all-gene tree, since there are few mutually exclusive sequences (Fig. 1.4). Single gene trees inferred from the 17 kDa surface antigen gene, *gltA*, and *sca4* resolved *R. parkeri* from Fairfax County However, bootstrap values are low (<70%). Surface antigens, which are subject to selective pressures, tend to be more appropriate for resolving phylogeny. For the remaining two genes, some loss in phylogenetic structure is observed. Phylogenetic analysis of *ompB* alone clustered FXVA sequences incorrectly with other *R. parkeri* sequences, despite a single nucleotide change shared by all samples. Analysis based on *ompA* grouped many of the *R. parkeri* sequences in an undifferentiated cluster. Single gene phylogenies including all other members of the spotted fever group (not shown) are supported by previous findings in the literature[50,52].

DISCUSSION

The major question addressed in this study was whether *R. parkeri* detection in *D. variabilis* ticks from Fairfax County, Virginia was the result of a single introduction by invasive *A. maculatum* followed by spillover into the resident tick population, rather than repeated introductions or previously undetected circulation. Multilocus sequence typing of partial *sca4*, *gltA*, *ompB*, and 17 kDa surface antigen precursor genes clearly demonstrate that *R. parkeri* nucleotide sequences from the landfill site are recognizably distinct. Further, phylogenetic analysis of five concatenated partial gene sequences confirm that samples collected from *A. maculatum* and *D. variabilis* at the landfill from 2012-2014 site exhibit a monophyletic relationship, with all other *R. parkeri* sequences available in GenBank basal to this association. These findings suggest that *R. parkeri*

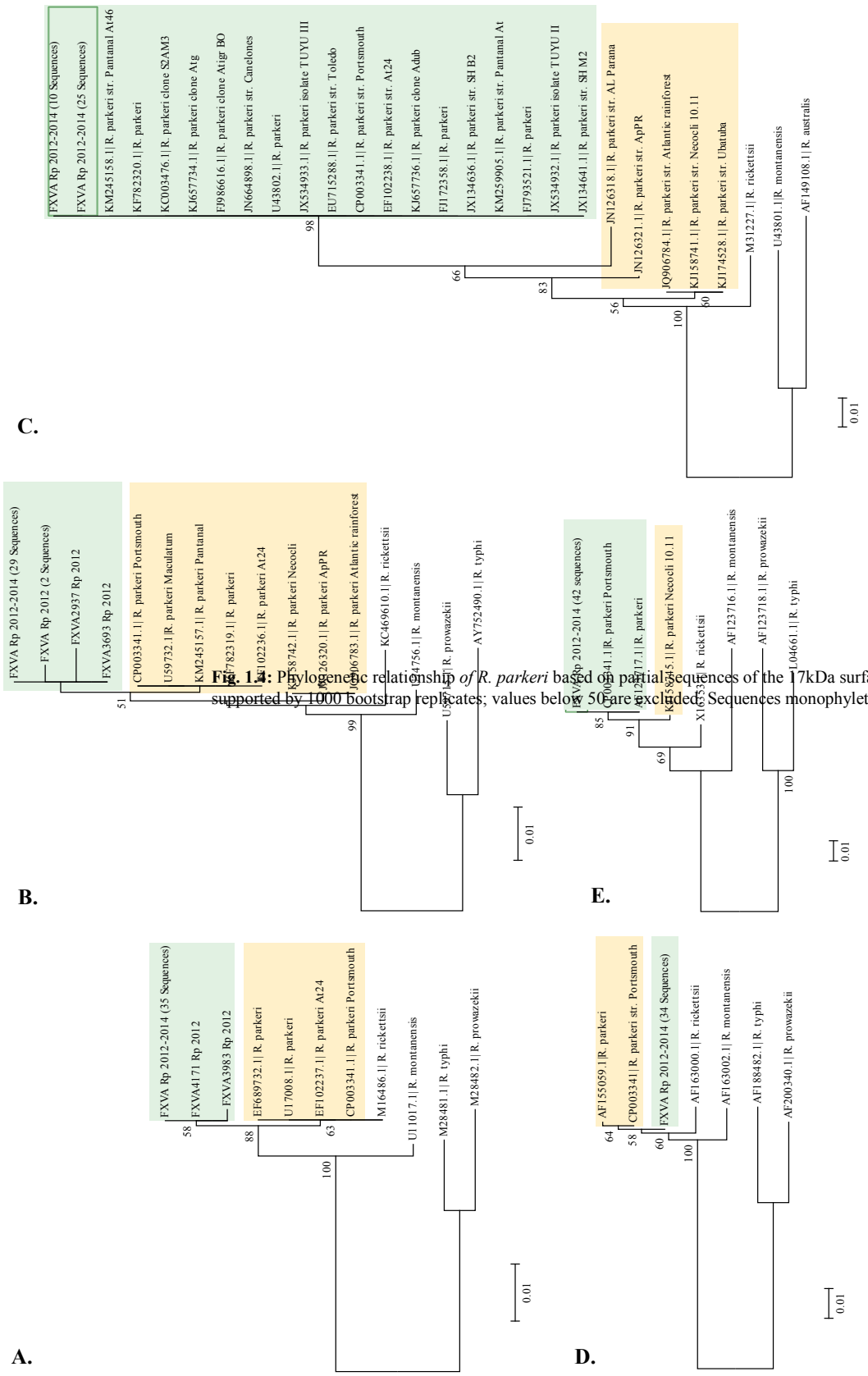


Fig. 1.4: Phylogenetic relationship *of R. parkeri* based on partial sequences of the 17kDa surface antigen gene (A), *gltA* (B), *o*
supported by 1000 bootstrap replicates; values below 50 are excluded. Sequences monophyletic with Fairfax County samples

infection in multiple tick species from one geographic focus in Fairfax County, Virginia resulted from one genetic variant introduced to the region.

Little genetic variability in the *ompA* gene was observed in *R. parkeri* from Fairfax County, regardless of tick species. This confirms the findings of several other studies of rickettsial diversity[45]. The *ompA* gene is the most variable gene shared by the spotted fever group rickettsiae, and is considered a standard target for speciation[56]. However, this study and others indicate that *ompA* analysis alone may not be sufficient, particularly for strain typing and characterizing genetic diversity of rickettsiae at the microscale[45,56,60,68]. Analysis of *gltA* proved most useful in distinguishing *R. parkeri* from Fairfax County, in direct contrast to studies noting that *gltA* has little power to differentiate between isolates of *R. prowazekii*[61]. Citrate synthase tends to be highly conserved across SFG rickettsiae. Although too few *R. parkeri* sequences are publically available to conduct an analysis, these findings suggest that *gltA* may be more variable among certain species and should not be overlooked in genetic analyses.

Additional information obtained from sequencing full genes, as opposed to gene fragments, would potentially further differentiate the pathogen circulating in this population from *R. parkeri* at other locales. Previous spotted fever group phylogenies relied on this method. Although the goal here was not to conduct a rigorous analysis of SFG relationships, this likely explains the minor discrepancies in phylogeny reported here. Although each mutation described in this study is expected to result in a functional protein, full gene sequencing would shed some light on this area. Additionally, a previous study of *R. conorii* indicated that sequencing of variable intergenic spacer regions (ISRs) might be more suitable than multigene sequencing for characterizing intraspecies genetic

diversity[60]. Further, phylogenetic clustering using this method was consistent with geographic distribution of strains[60]. Use of both techniques (*ompA* and ISR analysis) in *R. parkeri* from several tick populations in Mississippi and North Carolina found no genetic variation, but as previously noted, these populations lie well within the expected current range of *A. maculatum* in an *R. parkeri* endemic region, and pathogen similarity likely reflects the high degree of population mixing[45].

It would also have been informative to characterize the genetic diversity of the tick vector, particularly in regards to the *A. maculatum* population, alongside genetic analysis of the pathogen. Characterization of the 16S rDNA gene has previously been used to identify unique haplotypes within tick populations[,64], and comparison of established inland populations in Kansas and Oklahoma to coastal populations in Texas identified unique haplotypes associated with the two populations [46]. Newly established, isolated populations initiated by a few individuals (or, in the case of ticks, perhaps one individual) undergo the founder's effect, in which members of the population exhibit very low genetic diversity. In conjunction with the finding of low genetic diversity among *R. parkeri* from the landfill, such an analysis would provide additional support for the hypothesis that a newly established *A. maculatum* population introduced the pathogen to Fairfax County.

The range expansion and introduction of A. maculatum warrants a brief discussion. Although it is likely that a variety of wild and domestic animals are contributing to the range expansion of *A. maculatum*, some speculation is necessary. The relatively high nucleotide divergence of *R. parkeri* in Fairfax County from available sequences points to one of two things: first, the introduction could be from a relatively

distant geographic location, where sequence divergence might be more apparent; or second, that these sequences are representative of the degree of divergence one might expect at the forefront of expanding source populations. Mutations in populations at the edge of range expansions are more likely to be propagated in further population growth and expansion, resulting in both a higher allele frequency and greater distribution than expected in a fixed population[70].

As previously mentioned, birds are perhaps the most attractive hypothesis. *R. parkeri*-infected ticks are reported in infestations of migratory birds as far south as Brazil[71], and *A. maculatum* reported as far north as Canada[72]. Observations of ticks parasitizing humans from a military base in Tennessee observed that the re-establishment of migratory grassland bird species coincided with increased identification of *A. maculatum* ticks in the area, far beyond their historic range[5]. A study conducted in nearby Maryland during the same period as the landfill discovery (2008-2010) identified *A. maculatum* parasitizing 13 species of migratory songbirds[37], making it highly likely that birds are contributing to new populations along the mid-Atlantic.

In other locations, cattle, white-tailed deer, and feral swine have been implicated in the regional spread of *A.maculatum*. The beginnings of the exponential growth of white-tailed deer across the eastern US predate this study by several decades, although large resident populations of deer are almost certainly involved in local movements. In a 2010 survey, 282 specimens were removed from deer near the landfill site, although none were positive for *R. parkeri*[20]. Feral swine are also an intriguing possibility. Their northward range expansion approximates the estimated range of *A. maculatum* and individuals are capable of supporting large Gulf Coast tick infestations[5]. Feral pigs have

recently expanded into Virginia, and are reported in over 22 counties. Although not yet common in Fairfax County, the population epicenter is Culpeper County, less than 50 miles away[73]. It is possible that this and other focal populations of *A.maculatum* observed in Virginia were established by the movements of more local species, rather than migratory birds. Increased tick and host sampling efforts in Virginia and along the edge of *A. maculatum* expansion in conjunction with population genetic studies are needed to fully elucidate population movement events.

The landfill site was treated with permethrin twice in the summer of 2011, and once late in the summer of 2010, rapidly reducing the colonizing *A. maculatum* population to near zero. *A. maculatum* adult activity peaks in May, while larvae and nymphs peak in late summer. In contrast, *D. variabilis* adults peak in July in temperate regions, and larvae peaks are observed in both spring and late fall[1]. Thus, early and late summer treatments specifically targeted active *A. maculatum*, but had little effect on *D. variabilis* abundance. If the landfill site served as the source population for *R. parkeri*, treatment would be expected to hamper the spread of *A. maculatum* and *R. parkeri*. Supplementary collections during the year of 2011 indicated that *A. maculatum* might already have begun spreading prior to treatment, with infected ticks collected up to one mile away from the primary site[74]. These ticks were collected from greenspace areas directly adjacent to residential neighborhoods. However, no further follow-up has been performed to assess the degree of *R. parkeri* dispersal from the initial site. Regardless, infection of the resident *D. variabilis* population for four consecutive years following discovery of the infestation further suggest that *R. parkeri* is being maintained at the site. Additionally, low prevalence of *R. parkeri* in *D. variabilis* over time suggests that some

degree of horizontal transmission in unrecognized local hosts may be required for maintenance of the pathogen in tick populations[12].

The staggered phenology and host preferences of *A. maculatum* and *D. variabilis* make it equally plausible that spillover of *R. parkeri* into the *D. variabilis* population was the result of cofeeding or feeding on a rickettsemic host infected by *A. maculatum* earlier in the season. A number of associations implicate dogs as key hosts involved in the epidemiological expansion of *R. parkeri* in Fairfax County, Virginia. Both of the *Rh. sanguineus* and the *H. leporispalustris* ticks testing positive for *R. parkeri* were removed from dogs at a veterinary clinic in Fairfax County. Henning *et al*. speculated that this association was purely incidental—the ticks must have acquired the pathogen prior to attaching to their canine hosts[48]. However, the strong host preferences of *Rh. sanguineus* for dogs associate this species with kennel infestations, and they are infrequently collected from other environments[1]. Further, large carnivores, including domestic dogs, account for 53% of adult *A. maculatum* host composition, the remainder (42%) comprised of ungulates, such as white-tailed deer, cattle, and horses[31]. Adult *D. variabilis*—aptly named the American dog tick—are usually found on dogs[1]. In 2011, 6% of *D. variabilis* (2/33) collected by the Fairfax County Health Department from veterinary hospitals and animal shelters in Fairfax County tested positive for *R. parkeri*. Similar numbers (2/36) were obtained in 2012 (unpublished data).

Additionally, a survey found that 13% of domestic dogs in Louisiana animal control centers are infected with *R. parkeri*[75], and they represent the only animals in which *R. parkeri* rickettsemia has been confirmed in nature[5]. A growing body of evidence implicates canines as important hosts in the maintenance of several rickettsial

pathogens. Dogs are competent reservoirs for *R. conorii*, a closely related SFG *Rickettsia* transmitted by *Rh. sanguineus*[76]. Dogs also develop high *R. rickettsii* rickettsemia, although the ability of ticks to acquire the pathogen from infected dogs appears to be limited[77]. Very little else is known about potential reservoirs of *R. parkeri*. Experimental infection of two common hosts for immature *A. maculatum*, cotton rats and quail, demonstrated that quail were resistant to infection. Cotton rats developed rickettsemia, but ticks were unable to acquire bacteria from the species[78]. The limitations of this study preclude firm conclusions, but the overlapping hosts of *A. maculatum*, *D. variabilis*, and *Rh. rhipicephalus* and demonstrated ability of dogs to develop rickettsemia suggest a role for domestic dogs in the spillover of *R. parkeri* in Fairfax County, Virginia. Small sample sizes and very inconsistent sampling in veterinary settings introduce severe bias and limit the significance of the results. Further investigation of the role of dogs or other canines in the transmission cycle of *R. parkeri* is warranted, including the efficiency of transmission to naïve ticks of various species.

It is difficult to tease apart whether any sustained population infection or vector specificity is a result of *Rickettsia*-tick relationship or from ecological constraints imposed by host availability and geographic range. The evolutionary history of *Rickettsia* provides ample evidence for frequent switching to new vector lineages, particularly between closely related arthropods[70]. Although closely related rickettsae appear to inhabit related arthropods, mismatch in host and rickettsial phylogeny contradict the paradigm of coevolution. Some spotted fever group rickettsiae are clearly associated with a single tick species, such as *R. parkeri* with *A. maculatum*, while others, like *R. rickettsii*, are associated with ticks from multiple genera[79]. Widespread genome degradation appears to

be the most common signature of broad host changes, such as in the divergence of the tick-borne *R. masiliae* and *R. rickettsii* clusters from rickettsiae associated with other arthropods[79]. Further, intraspecies genotyping of the SFG has associated specific strains with different vectors. Characterization of six ISRs clearly associated 38 *R. rickettsii* isolates with different tick vectors implicated in outbreaks of RMSF, although specific features were not described[80]. Similarly, several genetic factors differentiate host-specific *R. felis* strains vectored by a variety of arthropods[62].

The limited availability of *R. parkeri* sequences from different tick species, both from this site and in GenBank, make it difficult to draw concrete conclusions about the genetic basis of spillover between tick species. Changes at the sequence level leading to adaptation to a particular vector could take place pre-shift, or after cross-species transmission as the pathogen responds to host selective pressures[29]. In this study, it is reasonably conceivable that the shifts in immunoexposed surface antigens PS120 (V459A) and rOmpB (V1226A) may be markers of pre-shift adaptation increasing the infectiousness of *R. parkeri* in *D. variabilis*. Both proteins are antigenic determinants in the *sca* family, a diverse group with important roles in host-parasite interaction[81]. This type of adaptation would be reflected in tests for non-neutral convergent evolution, although adaptive changes are unlikely to accumulate to detectable levels in such a short time and the dataset is limited temporally and geographically. Additionally, little information is available regarding the effects of specific mutations on the vector specificity of rickettsiae within the *R. rickettsii* cluster, and the evolutionary genetics of host shifts in arthropods more generally are poorly understood[55,79]. Whole genome

sequencing or characterization of additional gene targets, particularly those involved in host invasion, deserves more attention.

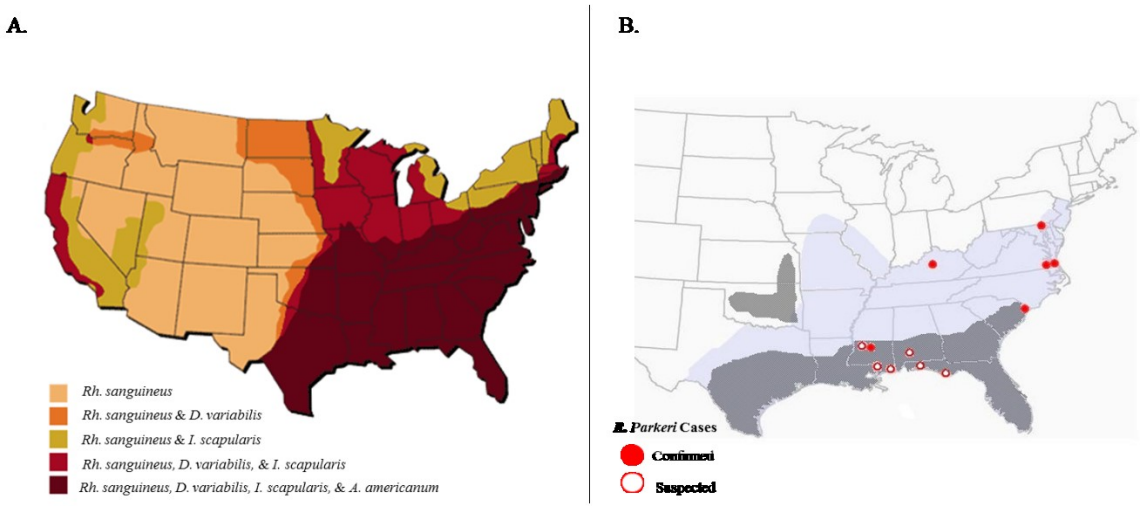


Fig. 1.5: (A) Distribution of common human-biting tick species in the US, excluding *A. maculatum*. Note that *Ixodes* (vector: Lyme disease) is included, but is not known to harbor disease-causing rickettsiase. Adapted from Mississippi Entomological Association. **(B)** Historic range (dark grey) of *A. maculatum* ticks. Tick populations throughout range expansion (light gray) are focal and often impermanent. *R. parkeri* cases (2002-2010) are shown[90].

One consequence of vector spillover is the potential for increased contact between *R. parkeri* and SFG rickettsiae harbored by invaded tick populations. Co-infection is an increasingly recognized phenomenon in tick biology, and *D. variabilis* simultaneously infected with *R. rickettsii*, *R. montanensis*, *R. bellii* (and an ancestral species) has been reported[79]. Co-infection in the same host likely plays a role in lateral gene transfer, and the majority of degraded genes in rickettsial genomes are thought to have been acquired this way[79]. Further, plasmids contribute to the evolution of rickettsiae and host-specific adaptation through genotypic plasticity[55]. Proteomic analysis of *R. parkeri* found no evidence for type IV secretion systems (T4SS), although the authors speculate that it may have been missed in the analysis; all other SFG rickettsiae encode some portion of the T4SS set[82]. No evidence for plasmids exists in the *R. parkeri* genome, demonstrating a

limited ability to undergo lateral gene transfer[79], although the number of plasmids within genomes of obligate intracellular organisms appears to be plastic and can be absent altogether[83]. Several papers speculate that *Rickettsia* may have the capability to exchange DNA by some yet-unrecognized mechanism, and acquisition of genetic material from the environment is certainly a possibility[83], so it is worth acknowledging the possibility of lateral gene transfer in the context of SFG co-infection. Effort should be made to address rickettsial co-infection in ticks, particularly in regions of increased contact between tick species, such as along range expansions.

Perhaps more importantly, overlap in the ranges of ticks known to harbor SFG rickettsiae has ecologic and epidemiological implications for SFG rickettsiosis. A complete lack of *R. rickettsii* has been observed in field collections across the southern states and mid-Atlantic[3,23,68,84], including those described here. A number of other SFG rickettsiae are known to circulate in mid-Atlantic sympatric tick species, including *R. montanensis*, *R. amblyomii*, and *R. parkeri,* the last of which now appears well-established in Virginia. A nearly 2.4-fold increase in the number of spotted fever rickettsiosis cases (per 100,000) reported in Virginia between 2010 and 2013[85] and correspondingly non-existent case fatality suggests a role for these pathogens in diagnosed spotted fever rickettsiosis cases. The contributions of each remains unknown. *R. amblyommii* and *R. montanensis* were recently implicated in mild pathology, but are not thought to cause febrile spotted fever[7]. In contrast, symptoms of *R. parkeri* rickettsiosis are sometimes indistinguishable from classic RMSF[86]. Rickettsiosis cases in Virginia have been increasing since well before the onset of this study, so it is plausible that *R. parkeri* has been circulating undetected in the region. Interestingly, several cases

in more recent years exhibited clustering near the Fairfax County landfill site[74]. It is highly speculative to state that these cases are the direct result of tick populations characterized in this study, but the association is noteworthy. The many assumptions made in this paragraph alone highlight the importance of continued vector and disease regions of spotted fever rickettsiosis endemnicity.

In general, this study resolves several questions about the origins of *Rickettsia parkeri* in Fairfax County, Virginia, and raises many more. The findings highlight the potential for *R. parkeri* dispersal and establishment of local transmission cycles following *A. maculatum* colonization of new geographic regions, and illustrate the value of longitudinal studies in characterizing founding populations. Influx of *R. parkeri*-infected *A. maculatum* has the potential to disrupt both local tick communities and the SFG rickettsiase composition within sympatric tick populations. Maintenance of *R. parkeri* in the local *D. variabilis* population for several years is notable*;* this species is rarely screened in surveillance studies, and perhaps represents an unrecognized potential reservoir for *R. parkeri*. Similarly, *R. parkeri* has been reported in *A. americanum* in Fairfax County[87]. In light of the recognized spillover and host-switching potential of SFG surveillance in understanding the complexity of tick-*Rickettsia* associations across rickettsiae, future surveillance studies should consider nontraditional vectors, and additional studies are needed to identify shared hosts and reservoirs. The changing ecology of SFG rickettsiae coinciding with increased reports of rickettsiosis in the mid-Atlantic warrants increased public health attention.

SPECIFIC FUTURE DIRECTIONS

In an attempt to address several unanswered questions within the bounds of this study, the concluding efforts will take several directions. *R. parkeri* has previously been identified in *A. americanum* ticks in Mississippi and Florida[18], and more recently, in Virginia[69,87]. The high rates of *R. amblyommii* in *A. americanum* (see Chapter 2 results) made our method of SFG rickettsiae determination impractical. However, exclusion of these samples eliminates a wealth of information about the ecology and epidemiology of *R. parkeri* at the landfill site. An essential next step will be to specifically screen *A. americanum*, and include PCR amplicons from those samples in the dataset, should any ticks be infected. To achieve this, a PCR assay using *R. parkeri*-specific primers for a fragment of the *ompA* gene (RpompAF and RpompRF) will be used to screen samples. The ability of these primers to distinguish between *R. amblyommii* and *R. parkeri* has been proven[47]. This will provide additional fine-scale resolution of infection patterns at the landfill site.

Effort will also be made to expand the geographic region of interest. A research team at Old Dominion University has been conducting tick-borne disease surveillance for several years, and has identified established *R. parkeri*-infected *A. maculatum* populations in southeastern Virginia. If a collaboration can be developed, it would also be of interest to apply the same methods and look for heterogeneity between multiple points across the state. This type of comparative analysis will further resolve the genetics of *R. parkeri* in new environments along the *A. maculatum* expansion front.

Lastly, a similar approach will be applied to the *A. maculatum* tick population. Bi-directional sequencing of the 16S mitochondrial rRNA gene is used to distinguish

between haplotypes of *A. maculatum*[46]. If the landfill population truly represents a founder population, ticks will exhibit haplotypes distinct from the surrounding region and little variability in haplotype will be observed among ticks collected at the site. Further, comparison of the haplotype(s) from Fairfax County to those obtained from studies in Arkansas[69], Mississippi, and North Carolina[45] may provide some insight into the location of the source population.

CHAPTER II

A brief report on other spotted fever group rickettsiae in Fairfax County, Virginia

THE STUDY

Multiple publications note that SFG rickettsiae play an important role in the regulation of *R. rickettsii* in its tick vector and RMSF transmission cycles in nature through the transovarial interference phenomenon. There exists a recognized need for increased understanding of the relationship between the prevalence of various SFG rickettsiae in tick populations and cases of spotted fever rickettsiosis. The previously described field and laboratory efforts intended to characterize *R. parkeri* introduction to Fairfax County, Virginia generated a significant amount of data about SFG rickettsiae in the region. Two other species, *R. montanesis* and *R. amblyommii*, were confirmed at the site.

The latter is harbored by *A. americanum* ticks. The causative agents of ehrlichiosis, tularemia, STARI (southern tick associated rash illness; etiologic agent unknown) are also carried by this species, and interestingly, its bite can induce allergy to red meat proteins. High rates of infection in *A. americanum* populations (up to 90%) across the USA and confirmed infection in larvae suggest that the bacterium is a fairly common, transovarially maintained endosymbiont. Implication in rash and apparent seroconversion in probable "RMSF" patients in North Carolina suggest that exposure to *R. amblyommii* may be the diagnostic cause of some reported rickettsiosis cases[6], although it is generally considered nonpathogenic. *A. americanum* is the most common tick removed from humans in the southeast and central United States. Although *R. amblyommii* is not necessarily the etiologic agent causing disease, frequent contact of these ticks with humans makes it highly likely that these bacteria are inflating estimated

SFG rickettsioses cases generated from serosurveys reliant on cross-reactive diagnostic tools.

Reports of *R. montanensis* infection in *D. variabilis* show that prevalence in most populations is below five percent—only 3.5% of ticks are typically infected, making it unlikely that *R. montanensis* is solely maintained by transovarial transmission. Supplemental maintenance in a zoonotic cycle is necessary; as is the case with *R. parkeri*, domesticated dogs and wildlife are suspected to play role[88]. A case of afebrile spotted rash in 2012 was conclusively linked to *R. montanensis*, although details of its pathogenicity remain largely unknown[7]. It is speculated that *R. montanensis* is responsible for some of the reported cases of spotted fever rickettsiosis, particularly in serosurveys.

This chapter provides a brief summary and initial analysis of other SFG rickettsiae in Fairfax County, Virginia. The method used to identify SFG rickettsiae to the species level in the previous chapter generated partial *ompB* sequence information for all *R. montensis*-positive ticks collected during 2014. These sequences were characterized, and *R. amblyomii* and *R. montanensis* infection prevalence is reported. A holistic examination of all spotted fever group rickettsiae in a particular region provides valuable information about how these bacteria interact in sympatry.

RESULTS

The prevalence of *R. montanensis* in *D. variabilis* in 2014 was 3.02% (Table 2.1). The proportion of infected *D. variabilis* appears to be increasing over time (Fig. S.1), although it was not specifically looked for in previous years and this could be a reflection

Table 2.1: Informative *R. montanensis* divergence from published sequences.

Target	Gene	Position	Nucleotide Change	Amino Acid Change
Citrate synthase II	*gltA*	509	T→C	Synonymous
17 kDa Surface Antigen	17 kDa precursor gene	242	G→A	Synonymous

of different screening procedures. No *R. montanensis* was detected in *A. maculatum*, nor has it been detected in previous years; sample size in 2014 was limited to nine samples. Genetic variability of *R. montanensis* in *D. variabilis* was assessed by sequencing 817 bp of the *ompB* gene. Complete identity was observed between *R. montanensis* from *D. variabilis* ticks at the landfill site and *R. montanesis* OSU 85-930 (GenBank accession no. CP003340). Partial *gltA* and 17 kDa surface antigen precursor gene sequences were evaluated to further assess genetic variability in a subset of two samples. Partial *gltA* and 17kDa surface antigen gene sequences were 99% similar to *R. montanensis* OSU 85-930, with the difference resulting from one synonymous 509T→C and one synonymous 242G→A nucleotide substitution, respectively (Table 2.1). When the phylogenetic relationship of SFG-wide sequences was inferred by the neighbor joining method (1000 bootstrap replicates) these two sequences cluster together with bootstrap values >98% (tree not shown).

In October 2010, 22.04% of *A. americanum* (67/304) were infected with *R. amblyomii.* No further assessments have been performed, and this remains an avenue for future investigation. High prevalence of *R. amblyommii* make it unlikely that other SFG rickettsiae be significantly maintained in the population. No *R. amblyommii* was detected in *D. variabilis* or *A. maculatum* in 2014, although infection was reported occasionally in *D. variabilis* in previous years (unpublished data).

Table 2.2: Other SFG rickettsiae in Fairfax County, 2014

Species	*D. variabilis*		*A. maculatum*		*A. americanum*	
	Total (Infected)	**Prevalence (%)**	**Total (Infected)**	**Prevalence (%)**	**Total (Infected)**	**Prevalence (%)**
R. montanensis	1389 (42)	3.02	9 (0)	0	NT	NT
R. amblyommii	1389 (0)	0	9 (0)	0	NT	NT
E. chaffeensis	-	-	-	-	1195 (26)	2.18

NT = not tested.

Multiple tick species are collected in Fairfax County and screened for a variety of pathogens, including *Ehrlichia*, *Anaplasma*, and *Borrelia*. After considering results, the prevalence of *Ehrlichia chaffeensis* is reported here, and is worth a brief discussion in the context of spotted fever group rickettsiosis (Table 2.2). Ehrlichiosis is a severe disease and can be fatal if not correctly and promptly treated. In the early stages, *E. chaffeensis* ehrlichiosis symptoms resemble RMSF, and rash is fairly common. It can be difficult to differentiate the two diseases based on clinical presentation alone, and as a result, some of the reported spotted fever cases may be ehrlichiosis[49,87]. Like spotted fever rickettsiosis and many tick-borne bacterial infections, doxycycline is the first line of defense, and antibiotics are often prescribed without confirming etiologic agent[49]. Two closely related pathogens, *Ehrlichia ewingii* and *Anaplasma phagocytophilum,* are also detected in Fairfax County ticks[87]. Infection with either is characterized by lack of rash and other symptoms are distinct from spotted fevers, so these pathogens are not discussed here.

DISCUSSION

This analysis identified some holes in the SFG rickettsiae dataset available for Fairfax County, VA. The future directions outlined to address *R. parkeri* spillover in *A.*

americanum should be expanded to include surveillance for *R. amblyommii*. At a 22%, the prevalence in *A. americanum* at the landfill has a much lower is lower than the 50-80% infection reported elsewhere in Virginia[87]. The "protective effect" conferred by transovarial interference is recognized[89], and reduced prevalence within the tick population may allow for the invasion of more pathogenic species, such as *R. parkeri* and *R. rickettsii,* that *A. americanum* is capable of vectoring. This prevalence estimate is based off a weeklong trap collection performed in the month of November, and its accuracy should be confirmed with multiple collections from more appropriate times during the field season.

The prevalence of *R. montanensis* in D. variabilis has increased steadily from 0.1% in 2010 to 3.7% in 2014 (Fig. S1) . Monitoring in the early years of the study was inconsistent, with surveillance often for nonspecific SFG rickettsiae only. For example, over 8% of ticks in 2009 were reported positive for SFG rickettsiae but no species differentiation was performed and the results are restricted to one monthly collection in September. It is possible that the unusually high prevalence reflects local infestation of an infected host. Previous years also utilized an *ompA* assay for screening samples, so it's possible that trend merely reflects differences in assay sensitivity. Results from 2014 are consistent with those reported elsewhere, and are likely a more accurate representation of *R. montanensis* circulation in this species in this region[68]. The apparent lack of *R. montanensis* in *A. maculatum* at this site is also interesting given that host and pathogen sharing appear to be common. Very low numbers of infected ticks make it statistically unlikely that naïve *A. maculatum* will come into contact with *R. montanensis*- infected *D. variabilis* in a way that will result in spillover, such as during co-feeding. However, as

previously discussed, *R. montanensis* is likely maintained in part by transmission cycles in animals, and identification of possible hosts would be of value.

In conclusion, *R. montanensis* and related pathogens appear to be well-established in Fairfax County, Virginia. It remains to be seen how the tick and pathogen population structure will change in light of the ongoing range expansion of multiple tick species, and ongoing surveillance is warranted.

CONCLUSION

The number of spotted fever group rickettsiosis cases have been increasing in the United States, complicated in part by misdiagnosis of various infections. This project and others suggest that it may be in part due to the expansion of *Amblyoma maculatum* ticks, which can establish new populations highly infected with *R. parkeri*. Further, *A. maculatum* range expansion brings these ticks into contact with a number of other species capable of harboring SFG rickettsiae, including *D. variabilis*, *A. americanum*, *Rh. sanguineus*, and *H. leporispalustris*. This study provides evidence for spillover of *R. parkeri* into *D. variabilis* in Fairfax County, Virginia following the establishment of an invasive *A. maculatum* population, and sustained *R. parkeri* circulation that persists despite acaricide treatment. Multiple tick species harboring various pathogens exist in sympatry in the county, and invasion of new species influence population structure and dynamics. Pathogen spillover into novel vectors could lead to increased transmission and propagation of the disease cycle, ultimately resulting in an increase in cases of spotted fever rickettsiosis. This study underscores and highlights the need for extensive, longitudinal surveillance for tick-borne pathogens.

Supplementary Data: Figures and Tables

The following pages contain supplementary data to aid in the understanding of Chapters 1 and 2 of this thesis. More detailed material illustrating tick collections, infection prevalence, and sequence alignments are provided.

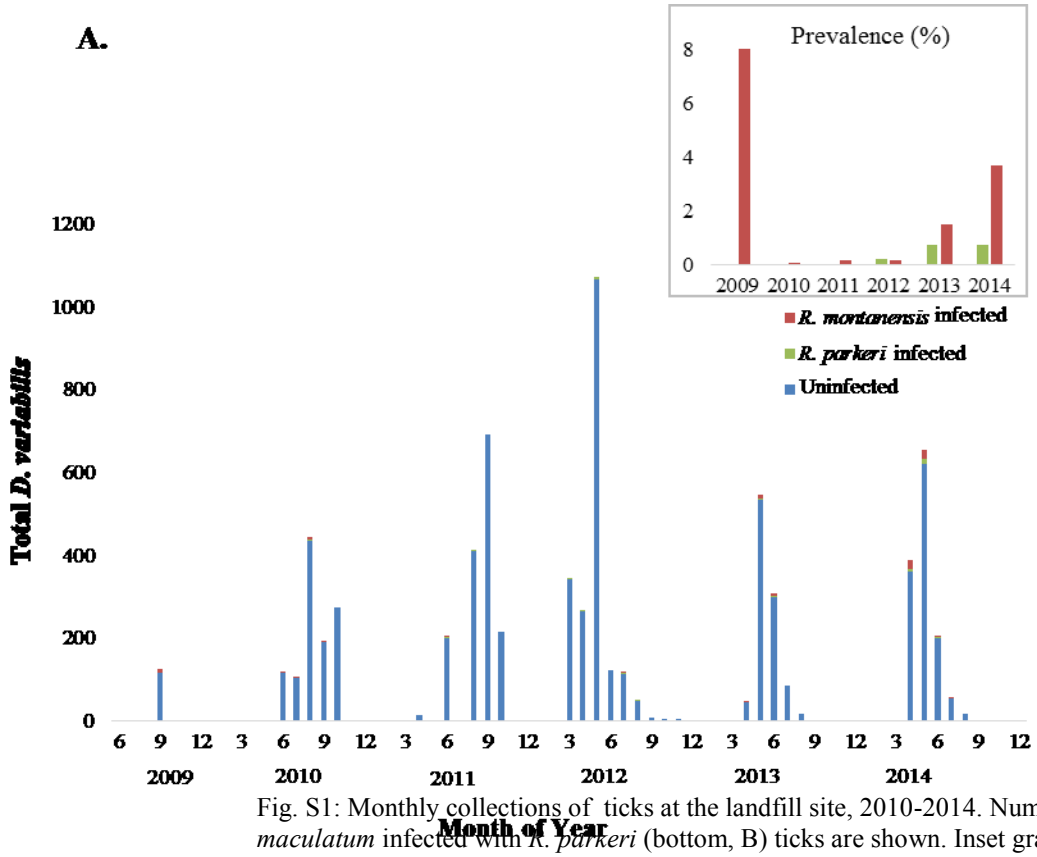
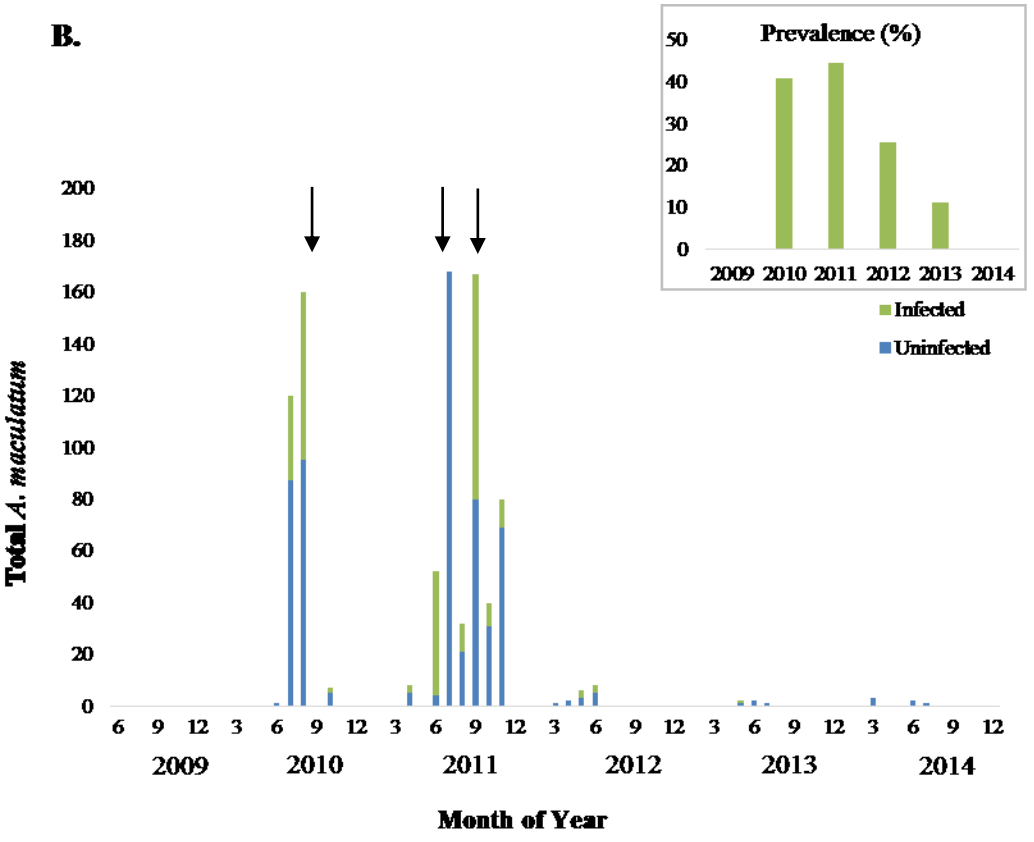


Fig. S1: Monthly collections of ticks at the landfill site, 2010-2014. Numbers of *D. variabilis* infected wi
maculatum infected with *R. parkeri* (bottom, B) ticks are shown. Inset graphs display yearly infection pre
treatments at the collection site. Note that collections in 2009 were not performed consistently and this pr



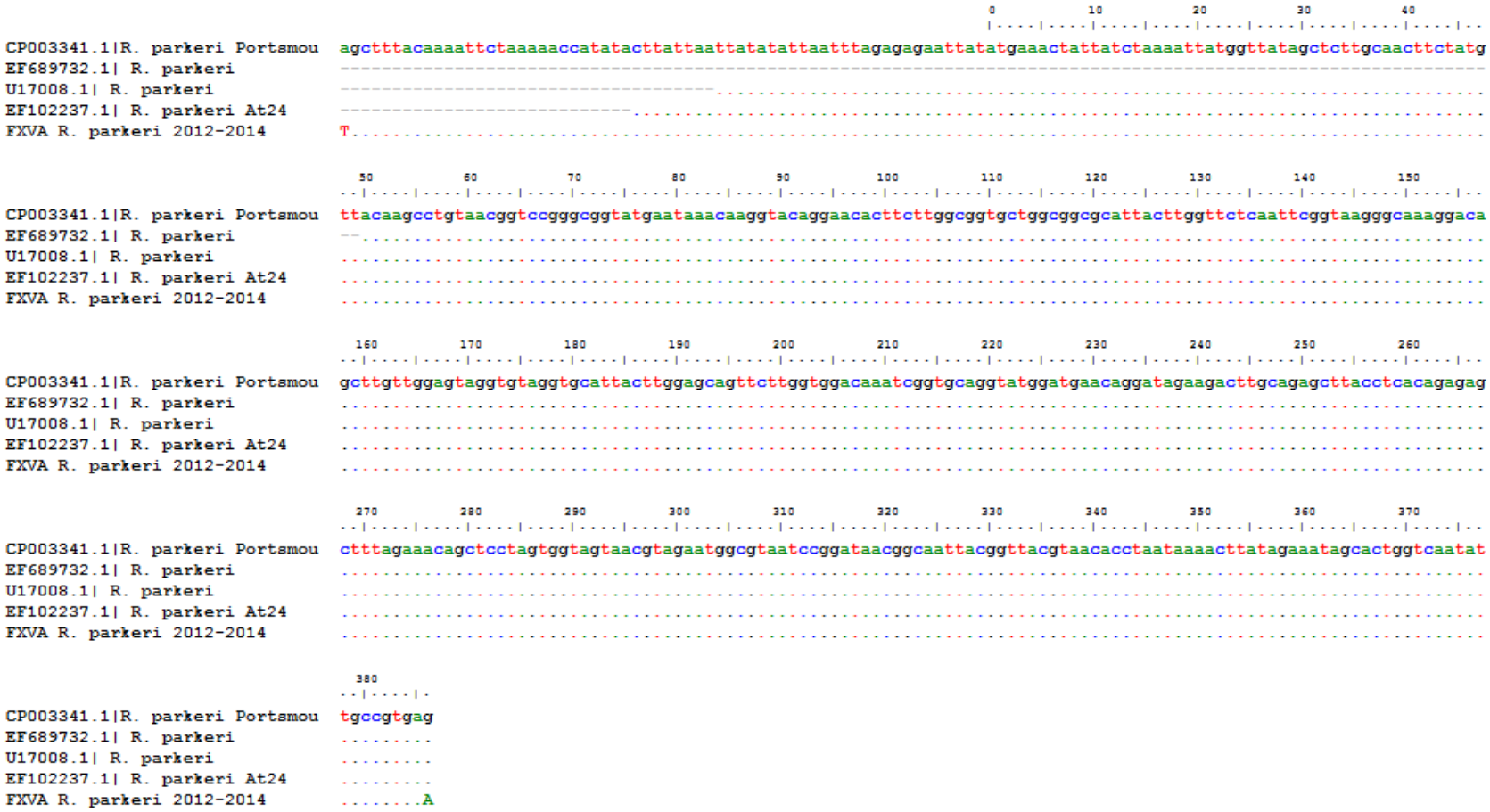


Fig. S2: Alignment of *R. parkeri* partial 17 kDa surface antiegen precursor gene sequences. Reference genome is *R. parkeri* Portsmouth (GenBank accession no. CP003341.1)

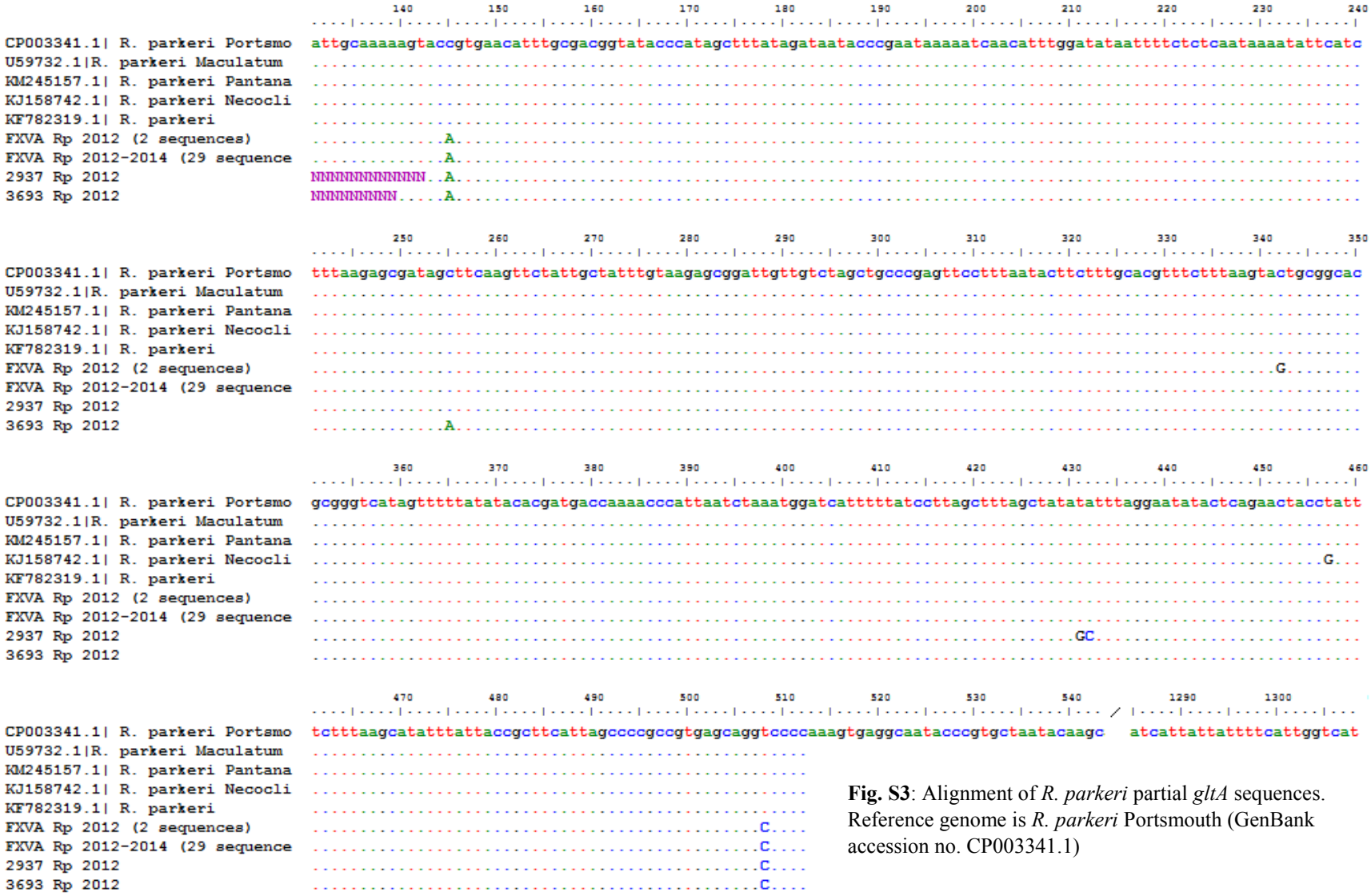


Fig. S3: Alignment of *R. parkeri* partial *gltA* sequences. Reference genome is *R. parkeri* Portsmouth (GenBank accession no. CP003341.1)

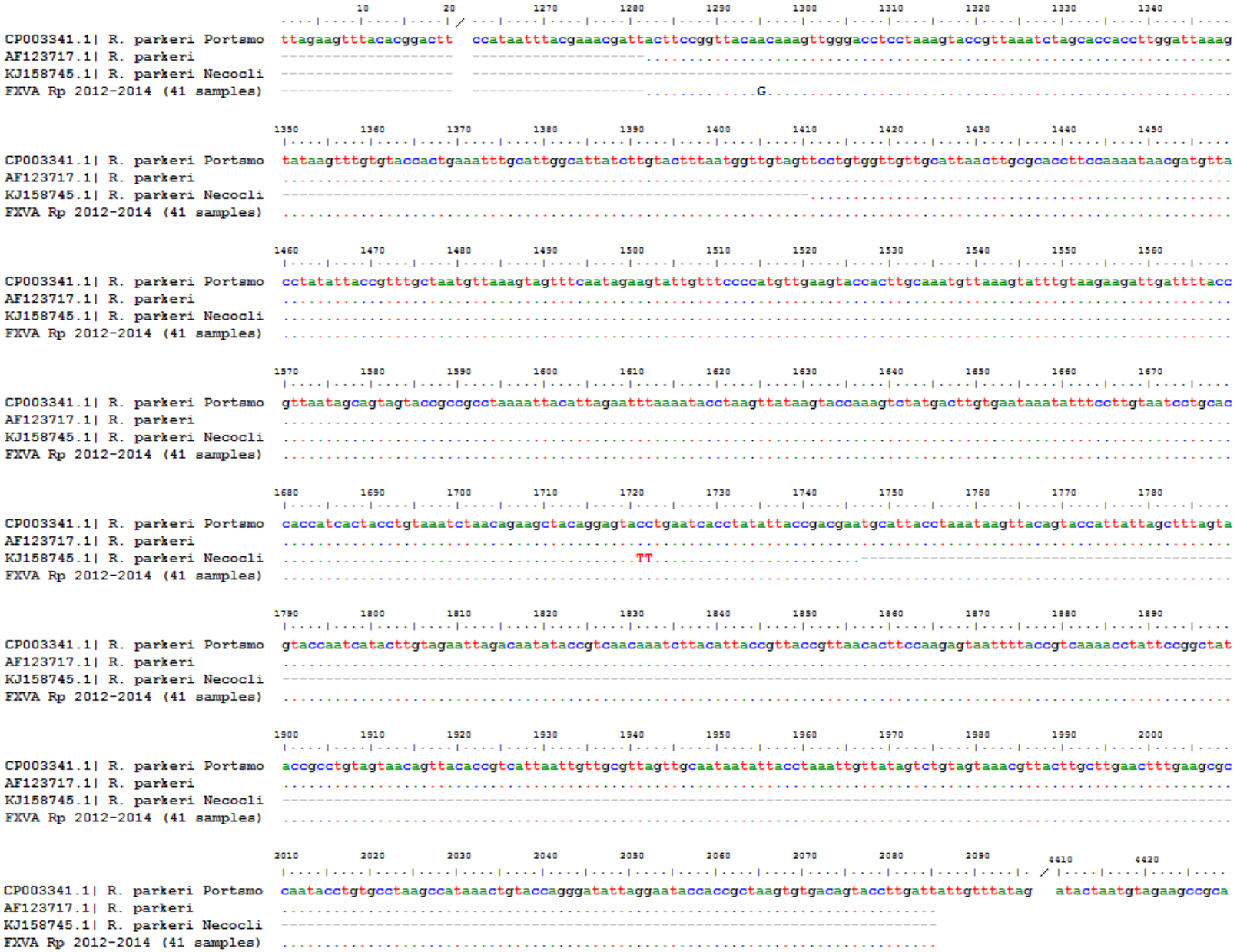

CP003341.1| R. parkeri Portsmo
AF123717.1| R. parkeri
KJ158745.1| R. parkeri Necocli
FXVA Rp 2012-2014 (41 samples)

Fig. S5 : Alignment of *R. parkeri* partial *sca4* sequences. Reference genome is *R. parkeri* Portsmouth (GenBank accession no. CP003341.1).

Fig. S4 (previous page): Alignment of *R. parkeri* partial *ompB* sequences. Reference genome is *R. parkeri* Portsmouth (GenBank accession no. CP003341.1)

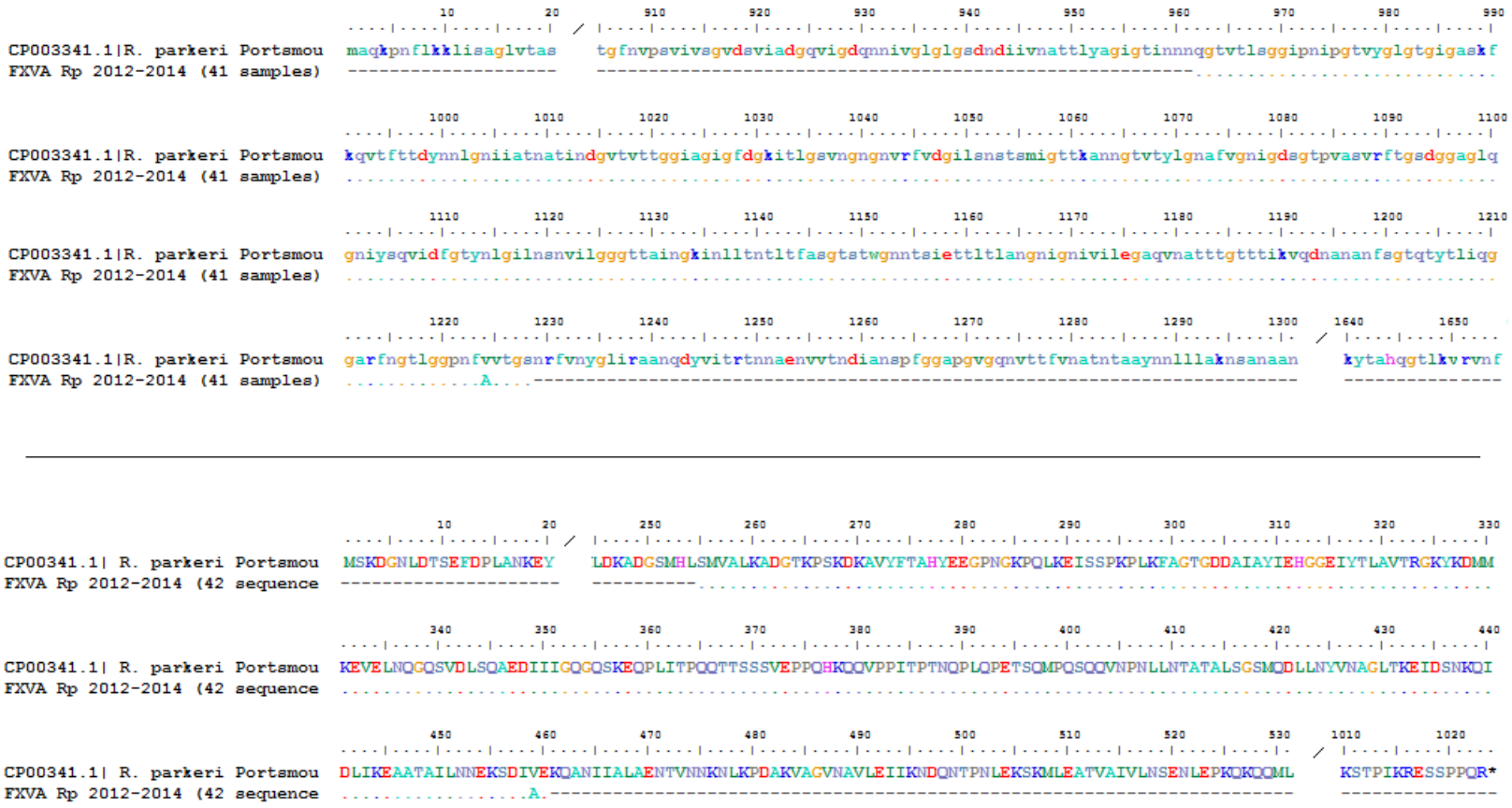


Fig. S6: translated amino acid sequence alignments of *R. parkeri* PS 120 and rOmpB. GenBank accession no. listed

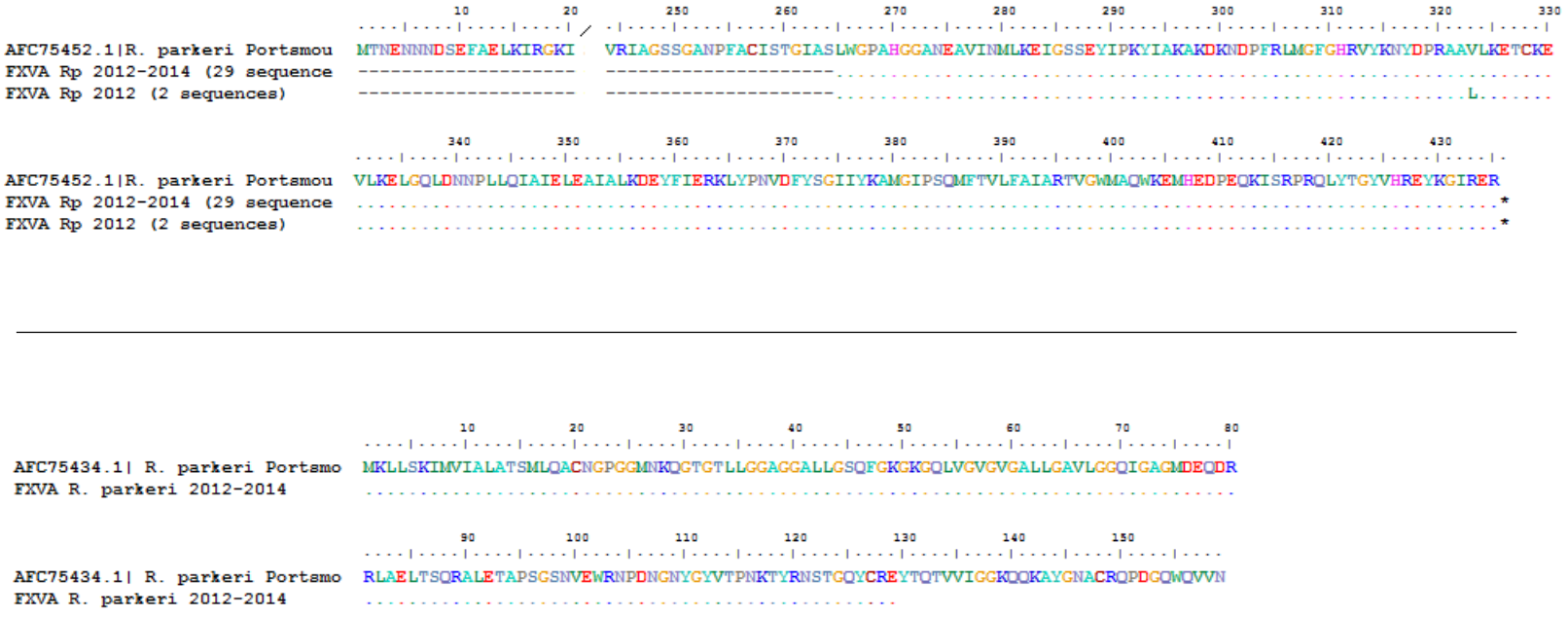


Fig. S7: Translated amino acid alignments of *R. parkeri* type II citrate synthase (top) and the 17 kDa surface antigen (bottom). GenBank accession no. listed.

Table S1: Subset of representative samples from Fairfax County, Virginia

Sample ID	Species	Year	Location Collected
FXVA 702	*D. variabilis*	2012	Landfill
FXVA 751	*D. variabilis*	2012	Landfill
FXVA 1002	*D. variabilis*	2012	Landfill
FXVA 2052	*D. variabilis*	2012	Landfill
FXVA 2433	*D. variabilis*	2012	Landfill
FXVA 2534	*D. variabilis*	2012	Landfill
FXVA 2927	*A. maculatum*	2012	Lorton
FXVA 2930	*A. maculatum*	2012	Landfill
FXVA 2931	*A. maculatum*	2012	Landfill
FXVA 2933	*A. maculatum*	2012	Landfill
FXVA 2934	*A. maculatum*	2012	Lorton
FXVA 2937	*A. maculatum*	2012	Lorton
FXVA 3681	*A. maculatum*	2012	Landfill
FXVA 3684	*A. maculatum*	2012	Landfill
FXVA 3692	*A. maculatum*	2012	Landfill
FXVA 3693	*A. maculatum*	2012	Lorton
FXVA 3694	*A. maculatum*	2012	Lorton
FXVA 3983	*D. variabilis*	2012	Landfill
FXVA 4011	*D. variabilis*	2012	Landfill
FXVA 4015	*D. variabilis*	2012	Landfill
FXVA 4171	*R. sanguineus*	2012	Landfill
FXVA 0110	*A. maculatum*	2013	Landfill
FXVA 1185	*D. variabilis*	2013	Landfill
FXVA 1561	*D. variabilis*	2013	Landfill
FXVA 1695	*D. variabilis*	2013	Landfill
FXVA 2000	*D. variabilis*	2013	Landfill
FXVA 2006	*D. variabilis*	2013	Landfill
FXVA 414	*D. variabilis*	2014	Landfill
FXVA 491	*D. variabilis*	2014	Landfill
FXVA 504	*D. variabilis*	2014	Landfill
FXVA 556	*D. variabilis*	2014	Landfill
FXVA 557	*D. variabilis*	2014	Landfill
FXVA 606	*D. variabilis*	2014	Landfill
FXVA 738	*D. variabilis*	2014	Landfill
FXVA 887	*D. variabilis*	2014	Landfill
FXVA 909	*D. variabilis*	2014	Landfill
FXVA 928	*D. variabilis*	2014	Landfill
FXVA 1045	*D. variabilis*	2014	Landfill

FXVA 1269	*D. variabilis*	2014	Landfill
FXVA 1270	*D. variabilis*	2014	Landfill
FXVA 1334	*D. variabilis*	2014	Landfill
FXVA 1503	*D. variabilis*	2014	Landfill

Samples are those collected 2012-2014 in Fairfax County that were PCR positive for all five gene targets. The only exception is FXVA 4171, from an *Rh. sanguineus* tick. Good sequence data was available for the 17 kDa surface antigen precursor gene.

Table S.2: Publically available *R. parkeri* and other SFG rickettsiae sequences used in analysis.

GenBank Accession No.	*R. parkeri* Strain	Location Collected	Tick Species
KC003476.1	*R. parkeri* clone S2AM3	Louisiana, USA	Unknown
KF782320.1	*R. parkeri*	Argentina	Unknown
cpoo3341	*R. parkeri* Portsmouth	USA	Unknown
FJ986616.1	*R. parkeri* clone Atigr_BO	Bolivia	*A tigrinum*
FJ986616.2	*R. parkeri* clone Atigr_BO	Bolivia	Unknown
EU715288.1	*R. parkeri* str Toledo	Uruguay	Unknown
U43802.1	*R. parkeri*	USA	Unknown
FJ172358.1	*R. parkeri*	Argentina	Unknown
JX134641.1	*R. parkeri* str SH_M2	USA	A maculatum
KJ158741.1	*R. parkeri* str Necocli 10.11	Columbia	A ovale
JQ906784.1	*R. parkeri* Atlantic rainforest	Brazil	Unknown
KJ657736.1	*R. parkeri* clone Adub	Uruguay	*A tigrinum A dubitatum*
KJ657734.1	*R. parkeri* clone Atg	Uruguay	*A tigrinum*
EF102238.1	*R. parkeri* At24	Brazil	Unknown
KM245158.1	*R. parkeri* str Pantanal At46	Brazil	*A triste*
JX534933.1	*R. parkeri* isolate TUYUIII	Argentina	*A triste*
JX534932.1	*R. parkeri* isolate TUYUII	Argentina	Unknown
KJ174528.1	*R. parkeri* Ubatuba	Sao Paulo, Brazil	*A ovale*
JN664898.1	*R. parkeri* str Canelones	Uruguay	Unknown
JN126321.1	*R. parkeri* ApPR	Brazil	ticks on birds
KM259905.1	*R. parkeri* Pantanal At	Brazil	Unknown
FJ793521.1	*R. parkeri*	Tennesse/Georgia, USA	Unknown
JN126318.1	*R. parkeri* AL Parana	Brazil	ticks on birds
JX134637	*R. parkeri* SH_B3	USA	*A maculatum*

GenBank Accession No.	*Rickettsia* Species	GenBank Accession No.	Species
KF646135.1	*R. aeschlimanii*	GU169051.1	*R. candidatus andeanae*
KF646135.1	*R. africae*	GU395298.1	*R. candidatus andeanae*
CP003375.1	*R. slovaca* 13-B	CP000847.1	*R. akari* Hartford
AE006914.1	*R. conorii*	CP0053.1	*R. felis* URRWXCal2
AP011533.1	*R. Japonica* YH	LN794217.1	*R. monacensis* IR/Munich
CP002912.1	*R. heilongjiangensis* 054	CP003340.1	*R. montanensis OSU 85-930*
CP003319.1	*R. massilae*	CP003342.1	*R. rhipicephali 3-7*
AY652981.1	*R. candidatus andeanae*	AF181036.1	*R. helvetica*
GU395298.1	*R. candidatus andeanae*	DQ365807.1	*R. raoultii*
KF179352.1	*R. candidatus andeanae*	JQ792108.1	*R. raoultii*
JQ792138.1	*R. raoultii*	HM050280.1	*R. sibirica*

KJ410264.1	*R. raoultii*	AF123722.1	*R. sibirica*
CP001227.1	*R. peacockii* Rustic	AF445384.1	*R. sibirica*
AF163004.1	*R. honeii*	CP004889.1	*R. prowazekii* Breinl
AF123724.1	*R. honeii*	CP003398.1	*R. typhi*
U43809.1	*R. honeii*	CP003338.1	*R. australis* Cutlack
AF018074.1	*R. honeii*	AF155057.1	*R. sibirica*
AF027124.1	*R. honeii*	JX945526.1	*R. sibirica*

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Appendix of Protocols

The following pages contain laboratory protocols used in the completion of Chapters 1 and 2 of this thesis.

Extraction of tick DNA using EPICENTRE MasterPure Complete DNA and RNA Purification Kit

1. Dilute 1 µL of 50 µg/ µL Proteinase K into 250 µL of 1X Tissue and Cell Lysis Solution for each sample. For 60 ticks, prepare master pure mixture.
    a. 1X Tissue and Cell Lysis Solution: 250 µL X 61 = 15250 µL
    b. 50 µg/ µL Proteinase K: 1 µL X 61 µL = 61 µL
    c. Take 251 µL master mixture for one tick
2. A tick is put into a 2 mL SealRite round-bottom tube with a 5-mm stainless steel bead and 50 µL Tissue and Cell Lysis Solution and then can be smashed by using the TissueLyser II at 30 HZ for 3 minutes.
3. Centrifuge the smashed ticks for 1 minute at 13200 RPM at room temperature in a microcentrifuge.
4. Add 251 µL of mixture from step 1 and then take the bead out by using a magnetic tool.
5. Mix thoroughly and incubate at 65 °C for 60 minutes; vortex mix every 10 minutes
6. Place the samples on ice for 3-5 minutes.
7. Add 150 µL of MPC protein Precipitation Reagent to 300 µL lysed sample and vortex mix vigorously for 10 seconds.
8. Pellet the debris by centrifugation at 4 °C for 10 minutes at 1400 RPM in a microcentrifuge.
9. Transfer the supernatant to a clean microcentrifuge tube and keep the pellet in freezer.
10. Add 500 µL of ice-cold isopropanol to the recovered supernatant. Invert the tube several (30-40) times.
11. Pellet the DNA/RNA by centrifugation at 4 °C for 10 minutes at 1400 RPM in a microcentrifuge.
12. Carefully pour off the isopropanol. Invert the tube and let the edge of the tube touch a paper towel to remove all isopropanol.
13. Rinse twice with 500 µL 75% ice-cold ethanol.
14. Air-dry your pellet and re-suspend the DNA/RNA in 30 µL of molecular grade water.

Nested rOmpA PCR

Primers:
190-70: 5'-ATG GCG AAT ATT TCT CCA AAA-3'
190-701: 5'-GTT CCG TTA ATG GCA GCA TCT-3'
190-FN: 5'-AAG CAA TAC AAC AAG GTC-3'
190-RN: 5'-TGA CAG TTA TTA TAC CTC-3'

PCR Program:
Outer (ROMPA-OU) 1hr 52min

1.	94°C	2 min
2.	94°C	15 sec
3.	60°C	30 sec
4.	72°C	45 sec
5.	Go to 2, X39	
6.	72°C	5 min
7.	4°C	forever

Inner (ROMPA-IN) 1hr 30min

1.	94°C	2 min
2.	94°C	15 sec
3.	55°C	30 sec
4.	72°C	45 sec
5.	Go to 2, X39	
6.	72°C	5 min
7.	4°C	forever

Reaction Mixture:

Outer	50 µL total volume
10X	5.0 µL
dNTPs 2.5 mM	2.0 µL (final conc. 100 µM each)
70P	0.5 µL (50 pmol)
701	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	40 µL

Use 1.5 µL of template DNA

Inner	50 µL total volume
10X	5.0 µL
dNTPs 2.5 mM	2.0 µL (final conc. 100 µM each)
FN	0.5 µL (50 pmol)
RN	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	40 µL

Use 2 µL of product from outer reaction

PstI *Rickettsia* spp. rOmpA RFLP

Amplicons from rOmpA-positive ticks can be used as template for this RFLP to determine species.

Reaction Mixture:	**25 µL**
10X NEBuffer 3	2.5 µL
100X BSA	0.3 µL
PstI endonuclease	1 µL
rOmpA PCR product	20 µL
dH_2O	1.2 µL

Incubate at 37°C for 1 hr

Reference:

Roux, V, Fournier, PE, Raoult, D. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. J Clin Microbiol 199; 34:2058-65.

Nested rOmpB PCR

Primers:
4362p Out: 5'-GTC AGC GTT ACT TCT TCG ATG C-3'
4836n Out: 5'-CCG TAC TCC ATC TTA GCA TCA G-3'
4496p In: 5'-CCA ATG GCA GGA CTT AGC TAC T-3'
4762n In: 5'-AGG CTG GCT GAT ACA CGG AGT AA-3'

PCR Program:
Outer (ROMPB-OU) 1hr 30min

1.	95°C	5 min
2.	95°C	15 sec
3.	54°C	15 sec
4.	72°C	30 sec
5.	Go to 2, 34X	
6.	72°C	3 min
7.	4°C	forever

Inner (ROMPB-IN) 1hr 30min

1.	95°C	5 min
2.	95°C	15 sec
3.	56°C	15 sec
4.	72°C	30 sec
5.	Go to 2, 34X	
6.	72°C	3 min
7.	4°C	forever

Reaction Mixture: 50 µL for Outer and 40 µL Inner Rxn

Outer

10X	5.0 µL
dNTPs 2.5 mM	2.0 µL (final conc. 100 µM each)
4362p	0.5 µL (50 pmol)
4836n	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	40.5 µL

Use 1 µL of template DNA

Inner

10X	4.0 µL
dNTPs 2.5 mM	1.6 µL (final conc. 100 µM each)
4496p	0.5 µL (50 pmol)
4762n	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	32.1 µL

Use 1 µL of product from outer reaction

Rickettsia spp. rOmpB PCR
This PCR protocol amplifies a ~800 bp fragment of the *ompB* gene encoding a surface antigen.

Primers:

120-2788: 5'-AAA CAA TAA TCA AGG TAC TGT-3'

120-3599: 5'- TAC TTC CGG TTA CAG CAA AGT-3'

PCR Program: CDC ompB,

1.	95°C	3 min
2.	95°C	30 sec
3.	50°C	30 sec
4.	68°C	1.5 min
5.	Go to 2, 39X	
6.	68°C	7 min
7.	4°C	forever

Reaction Mixture:

Outer	50 µL total volume
10X	5.0 µL
dNTPs 2.5 mM	2.0 µL (final conc. 100 µM each)
4362p	0.5 µL (50 pmol)
4836n	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	40.5 µL
Template DNA	1.0 µL

***Rickettsia* spp. Gene D PCR**

This diagnostic amplifies a segment (~600bp) of *sca4* ('gene D'), which encodes an intracytoplasmic protein.

Primers:
D767f: 5'-CGA TGG TAG CAT TAA AAG CT-3'
D1390r: 5'-CTT GCT TTT CAG CAA TAT CAC-3'

PCR program: GENED-RI, 2:45 hr

1.	95°C	5 min
2.	95°C	15 sec
3.	54°C	15 sec
4.	72°C	30 sec
5.	Go to 2, 34X	
6.	72°C	3 min
7.	4°C	forever

Reaction Mixture:

10X	5.0 µL
dNTPs 2.5 mM	2.0 µL (final conc. 100 µM each)
4496p	0.5 µL (50 pmol)
4762n	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	32.1 µL
Template DNA	1.0 µL

17kDa *Rickettsia* spp. PCR
This semi-nested PCR protocol amplifies part of the Rickettsia 17kDa cell surface antigen gene, producing a product of 435 bp.

Primers:
17kD5': 5'-GCT TTA CAA AAT TCT AAA AAC CAT ATA-3'
17kD3': 5'-CTT GCC ATT GTC CRT CAG GTT G-3'
17kD3'nest: 5'-TCA CGG CAA TAT TGA CC-3'

PCR Program:
Outer (17KD-OUT) 3hr 8min

1.	95°C	5 min
2.	95°C	1 min
3.	53°C	1 min
4.	60°C	1.5 min
5.	Go to 2, 34X	
6.	60°C	15 min
7.	4°C	forever

Inner (17KD-INN) 3hr 8min

1.	95°C	5 min
2.	95°C	1 min
3.	56°C	1 min
4.	60°C	1.5 min
5.	Go to 2, 34X	
6.	60°C	15 min
7.	4°C	forever

Reaction Mixture:

Outer	50 µL total volume
10X	5.0 µL
dNTPs 2.5 mM	2.0 µL (final conc. 100 µM each)
4362p	0.5 µL (50 pmol)
4836n	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	40.5 µL
Template DNA	1.0 µL

Inner	25 µL total volume
10X	4.0 µL
dNTPs 2.5 mM	1.6 µL (final conc. 100 µM each)
4496p	0.5 µL (50 pmol)
4762n	0.5 µL (50 pmol)
Taq	0.5 µL (2U)
dH_2O	32.1 µL
Outer rxn product	1.0 µL

Maureen K. Kessler

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EDUCATION

Montana State University, Bozeman, MT — Expected May 2020
Doctor of Philosophy in Microbiology and Immunology
Accepted beginning fall 2015
Research interests: bat-borne zoonotic diseases, ecology, land-use change, modelling.

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD — Expected May 2015
Master of Science in Molecular Microbiology and Immunology
Thesis: Genetic Diversity of *Rickettsia parkeri* in Sympatric Tick Populations at a Focal Site in Northern Virginia

New Mexico Institute of Mining and Technology, Socorro, NM — Conferred May 2013
Bachelor of Science in Biology with Highest Honors, chemistry minor

University of Bradford, Bradford, Yorkshire, United Kingdom — August 2012 – January 2013
Study Abroad - Biomedical Sciences

EXPERIENCE AND EMPLOYMENT

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Graduate Research – Department of Molecular Microbiology and Immunology — October 2013 – present
Advisor: Dr. Douglas E. Norris

- Performing routine surveillance for Spotted Fever Group rickettsiae in field-collected ticks.
- Characterizing genetic diversity and phylogenetic relationship of several *Rickettsia parkeri* gene targets.
- Processing field-collected mosquitoes for molecular species identification, blood meal analysis, and malaria infection; will travel to Zambia in March to continue work in the field.

Independent Study – Department of Epidemiology — March 2013 – present
Mentors: Dr. Derek Cummings and Dr. Justin Lessler

- Continuation of course project modeling the shared seasonality and dynamics of childhood diseases in the pre-vaccine era across major US cities using the statistical computing software R; publication anticipated.

Sandia National Laboratories, Albuquerque, NM — April 2011 – August 2013
Technical Research Intern – Bioenergy and Defense Technologies
Principle Investigator: Dr. Jerilyn A. Timlin

- Examined virus-host interaction using time-lapse hyperspectral confocal fluorescence microscopy.
- Evaluated photosynthetic and metabolic changes using PAM fluorometry, spectroscopy, flow cytometry, and oxygen evolution measurements to identify early signatures of viral infection.
- Held a Department of Energy security clearance; publication in preparation.

New Mexico Institute of Mining and Technology, Socorro, NM — January – May 2013
Undergraduate research – Departments of Biology
Principle Investigator: Dr. Rebecca A. Reiss

- Assessed the effects of a high fat diet on methylation patterns in rat tissue using bisulphite sequencing and molecular techniques.

Socorro General Hospital, Socorro, NM — February 2009 – October 2011
Registrar – Registered patients and collected insurance copays; shadowed physicians; volunteered for laboratory.

TEACHING AND MENTORING

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD — Spring 2015

Teaching assistant – 260.650, Vector Biology & Vector-borne Disease. Professor: Dr. Douglas E. Norris

STEM Achievement in Baltimore Elementary Schools, Baltimore, MD August 2014 – present
Mentor – Guided classroom science activities and mentored students to promote early STEM education.

PUBLICATIONS AND PRESENTATIONS

Kessler MK, Henning TH, Norris DE. *Rickettsia parkeri* dispersal across sympatric tick populations in northern Virginia. (Oral) Mid-Atlantic Tick Summit IV, Feb. 2015; Laurel, MD.

Kessler MK, Henning TH, Orr JM, Smith JD, Arias JR, Norris DE. Spillover and genetic diversity of *Rickettsia parkeri* in northern Virginia. (Poster) *American Society of Tropical Medicine and Hygiene 63rd Annual Meeting*, Nov. 2014; New Orleans, LA.

Das S, Muleba M, Eng S, **Kessler MK**, Norris DE. Seasonality of multiple blood feeding behavior in anopheline mosquitoes and implications for malaria transmission in Nchelenge District, Zambia (Poster) *American Society of Tropical Medicine and Hygiene 63rd Annual Meeting*, Nov. 2014; New Orleans, LA.

Kessler MK, Collins AM, Jones HDT, Carney LT, Lane TW, Timlin JA. 2012. Tracking early infection events of the *Chlorella* virus PBCV-1 with hyperspectral confocal microscopy. *Microscopy and Microanalysis*, 18 (Suppl. 2): 226-227. doi:10.1017/S143192761200298X.

Kessler MK, Bryant SR, Baeza M, Tsosi J, Eubank L, Ogunro C, Reiss RA. Methylation of CpG islands near noncoding RNAs upregulated in high-fat diets. (Poster) *New Mexico Bioinformatics, Science, and Technology Symposium*, March 2013; Santa Fe, NM.

HONORS AND AWARDS

Molecular Biosciences Fellowship, Montana State University	2015 – 2016
Master's Tuition Scholarship, JHBSPH	2014 – 2015
EcoHealthNet Workshop & Travel Award, EcoHealth Alliance ($300)	August 2014
Student Conference Fund Travel Award, JHBSPH ($350)	April 2014
First Prize Microscopy Society of America Student Poster Award in Biological Sciences ($400)	July 2012
New Mexico Tech Fifth Year Scholarship ($2000)	2012 – 2013
New Mexico Tech Scholar	2009 – 2013
New Mexico Tech Academic Honor Roll	2008 – 2013
New Mexico Tech Silver Academic Scholarship	2008 –2012
Tome Community Association Scholarship ($500)	2008 – 2009

LEADERSHIP AND SERVICE

Social Chair, Molecular Microbiology and Immunology Student Group, JHBSPH	August 2014 – present
New Student Mentorship Program, Molecular Microbiology and Immunology, JHBSPH	August 2014 – present
Member, Lab Sciences Student Group, JHBSPH	April 2014 – present
National MS Society volunteer, Pedal Los Pueblos bike MS150	Summer 2013
Volunteer, Cycling4All Disability Cycling Club	August 2012 – January 2013
Volunteer, Sandia National Laboratories Volunteer Outreach Program	2011 – 2013
Volunteer, Socorro General Hospital	August – December 2011
Judge, New Mexico State Science and Engineering Fair	March 2011
Vice President, Venture Scouts, Los Lunas Crew	2008 – 2010

PROFESSIONAL AFFILIATIONS

American Public Health Association (APHA), student member	
American Association for the Advancement of Science (AAAS), student member	2010 – 2013
Beta Beta Beta National Biological Honor Society	2009 – 2012